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Study of Hip1r: Insights from a *Dictyostelium discoideum* Clathrin Adaptor

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Study of Hip1r: Insights from a *Dictyostelium discoideum* Clathrin Adaptor

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Dedication

To James and John

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Study of Hip1r: Insights from a *Dictyostelium discoideum* Clathrin Adaptor

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The transport of material across the plasma membrane and between subcellular compartments is a major trafficking event by which cells communicate, regulate developmental processes and maintain homeostasis. Clathrin-coated vesicles select and traffic proteins to specific compartments in eukaryotic cells. Recently a large number of proteins have been identified that serve as clathrin adaptors and accessory proteins. Information regarding the interaction between individual clathrin adaptors and accessory proteins during coated pit formation is currently lacking. Here we investigated the intracellular role of one clathrin adaptor, *Dictyostelium discoideum* Hip1r, and identified a functional relationship between Hip1r and a second clathrin adaptor, epsin. Hip1r is phosphorylated and localizes to punctae on the plasma membrane that also contain epsin. Moreover, expression of the NH_2-terminal ENTH domain of epsin alone was sufficient to restore both the phosphorylation and the restricted localization of Hip1r to the plasma membrane. Analysis of the individual Hip1r domains demonstrated the phosphorylation event is also dependent upon the expression of the central coiled-coil region of the Hip1r. During development, Hip1r null cells form mature fruiting bodies that yield defective spores. While the

mutant spores contain both cellulose and at least one protein secreted from prespore vesicles, spore coats lack the organized fibrils typical of wild type spores. Moreover, Hip1r spores are round, rather than ovoid, and exhibit decreased viability. Domain analysis of Hip1r in conjunction with investigation of phenotypes associated with a Hip1r/epsin double mutant reveal a requirement for full length Hip1r in the production of robust spores. Results from this study suggest that the Hip1r protein functions with epsin during cellular events in both growing and developing *Dictyostelium* cells and reveals a previously unidentified interaction between two clathrin adaptors.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1 THE SIGNIFICANCE OF CLATHRIN-MEDIATED ENDOCYTOSIS

Regulating transport across cellular membranes is of utmost importance in every living organism, from the single-celled amoeba to the multi-cellular mammal. The importance of this biological process is underscored by the existence of an assortment of pathways designed to accomplish the uptake of material from the surrounding environment by individual cells. Clathrin-mediated endocytosis, defined as the transfer of material into the cell via budding of clathrin-coated vesicles from the plasma membrane, is a major route of cellular internalization for eukaryotic cells (Kirchhausen, 2000; Brodsky et al., 2001). While cells employ other pathways independent of clathrin to internalize extracellular substances, the present study will focus on proteins associated with clathrin-dependent processes.

The importance of clathrin-dependent endocytosis is illustrated by the pathologies that occur when endocytosis is disrupted. Studies of potential causes of hypercholesterolemia played a major role in defining the process of receptor-mediated endocytosis. Hypocholesterolemia is a condition that arises when low density lipoprotein (LDL) is not internalized into cells and instead remains in the blood. This defect can be caused by defective endocytosis of the LDL receptor by the clathrin pathway (Anderson, et al., 1977). More recently, researchers have identified clathrin-mediated endocytosis as the mode of entry of hepatitis C virus and influenza virus into the cell (Blanchard, et al., 2006; DeTulleo and Kirchhausen, 1998).

In addition, a number of neurodegenerative diseases develop when proteins that function during clathrin-dependent processes are perturbed, most likely because of the importance of the pathway in synaptic transmission. Studies with patients with Alzheimer's disease have demonstrated a decrease in expression of two major clathrin adaptor proteins, the AP-2 complex and AP180/CALM (Yao et al., 1999; Yao et al., 2000). Impaired function of other clathrin adaptors, such as the Hip1r orthologs, also leads to disorders of the nervous system. Huntington's disease is a devastating neuropathy caused by the mutation of the huntingtin protein (Vonsattel and DiFiglia, 1998). In the mammalian brain, the huntingtin protein interacts directly with HIP1 (Huntingtin Interacting Protein 1) (Kalchman et al., 1997). Hip1 has been shown to modulate the activity of NMDA receptors, receptors present at neuronal synapses that have a role in synaptic plasticity and excitotoxic cell death (Metzler, et al., 2007). The function of these receptors appears to be enhanced in Huntington's disease (Vonsattel and DiFiglia, 1998). Hip1, in turn, directly interacts with Hip1r (Huntingtin Interacting Protein 1 Related) (Legendre-Guillemin et al., 2005) Analysis of human Hip1r mutants exhibiting a number of tandem repeat polymorphisms may be involved in the onset of bipolar disorder (Provençal et al., 2004). Both Hip1 and Hip1r belong to a small family of proteins, the Sla2/Hip1 family, that have been shown to act during clathrin-mediated endocytosis in a variety of model systems (Henry et al., 2002; Newpher and Lemmon, 2006; Mishra et al., 2001; Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2005). The *Dictyostelium discoideum* member of this family, *Dictyostelium* Hip1r, is the focus of the present study.

1.2 CLATHRIN

The first description of clathrin-coated vesicles came in 1964 from Thomas Roth and Keith Porter who were studying the internalization of yolk proteins by mosquito oocytes (Roth and Porter, 1964). Years later, using purified brain extracts, Barbara Pierse identified the major component of clathrin coats, the clathrin heavy chain (Pearse, 1975). In mammals, clathrin heavy chain is a 190 kilodalton (kDa) protein that is composed of three regions, the proximal, distal and terminal region (Kirchhausen 2000; Brodsky et al., 2001). The basic unit of a clathrin coat is the triskelion, a trimeric assembly of three clathrin heavy chains each with their own associated clathrin light chain (Kirchhausen, 2000; Brodsky et al., 2001). Clathrin light chains are smaller subunits that bind, noncovalently, to the proximal terminus of the heavy chain and influence assembly of the clathrin coat (Brodsky et al., 1991; Wang et al., 2003). The carboxyl (proximal) termini of the clathrin heavy chains join together to form the vertex, or hub, of the clathrin triskelion (Kirchhausen, 2000; Brodsky et al., 2001). The legs from adjacent triskelions intertwine and form lattices on the plasma membrane prior to pit formation. As the membrane invagination grows, so does the clathrin coat. However, clathrin alone is not sufficient for formation of the lattice on the membrane or for subsequent steps involving endocytosis. A myriad of proteins assist in the formation and release of clathrin-coated vesicles (Kirchhausen, 1999; Sorkin, 2004; Owen et al., 2004; Maldonado-Báez and Wendland, 2006). Clathrin accessory proteins have many important roles in endocytic events; from promoting clathrin assembly and sorting of receptors into the coated pits to release of the mature vesicle from the plasma membrane.

1.3 CLATHRIN-MEDIATED ENDOCYTOSIS

1.3.1 Overview

Clathrin-dependent endocytosis is a dynamic process involving an assortment of proteins, membrane and filamentous actin in eukaryotic cells (Kaksonen et al., 2003, Owen et al., 2004; Smythe and Ascough, 2005). While variations exist between organisms, the basic model of clathrin-dependent events can be described as follows. Clathrin is recruited by clathrin adaptor/assembly proteins such as AP2 and AP180 to sites of the membrane where endocytosis will occur (Kirchhausen, 1999, Brodsky et al., 2001; Owen et al., 2004; Maldonado-Báez and Wendland, 2006). Polymerization of clathrin triskelions forms a coated pit and drives membrane invagination. Cargo fated to be internalized is concentrated in the pit with the help of adaptor proteins. Through a series of complicated protein and lipid interactions, the coated pit further invaginates to form a thin "neck" of membrane (Ford et al., 2002; Legendre-Guillemin et al., 2004). Dynamin, a GTPase, binds this neck region and acts to sever the coated pit from the plasma membrane (Hinshaw, 2000). In addition, the actin cytoskeleton appears to have a role in vesicle scission and release (Engqvist-Goldstien and Drubin 2003; Merrifeld, 2004; Yarar et al., 2005). Once separated from the membrane, the structure is no longer a pit but a clathrin-coated vesicle containing cargo to be delivered to early endosomes and eventually disseminated to various parts of the cell. After scission but before fusion with the endosomal system, the vesicle sheds the clathrin coat (Brodin et al., 2000). The successful completion of the internalization process necessitates a number of interactions between clathrin and associated accessory proteins, as well as interactions between the accessory proteins themselves. However thus far, information regarding potential interactions between individual accessory proteins during clathrin-mediated events is significantly lacking.

1.3.2 Clathrin Associated Proteins: Clathrin Accessory Proteins, Clathrin Assembly Proteins and Clathrin Adaptors

Since the identification of clathrin and subsequent analyses of clathrin-mediated endocytosis, the discovery and characterization of proteins associated with clathrin has dominated the area of endocytic research. The classification of factors involved in clathrin-dependent events is necessary to clarify nomenclature within the field as well as to appreciate the contribution of individual proteins to vesicle formation. Proteins involved in the construction and maturation of clathrin-coated vesicles may be categorized as clathrin accessory proteins, clathrin assembly proteins or clathrin adaptors. All proteins associated with clathrin during membrane trafficking events, whether they act in the early or late stages of endocytosis, can be considered clathrin accessory proteins. Clathrin assembly proteins can be defined as clathrin binding proteins that act to recruit clathrin to the membrane, promote polymerization of the clathrin lattice and support coated pit formation. Finally, the definition of clathrin adaptors is less straight forward and differs between research groups engaged in characterization of such proteins (Owen et al., 2004; Maldonado-Baez and Wendland, 2006). For the purposes of this study, I will define a clathrin adaptor as a protein that links clathrin triskelions or assembled clathrin, either through protein or lipid interactions, to the cytoplasmic face of the plasma membrane. A number of clathrin adaptors function as cargo adaptors by binding the cytoplasmic tails of transmembrane receptors to promote their incorporation into a vesicle. In terms of classification, some proteins, such as those involved in vesicle scission or vesicle uncoating, are defined as members of only one category, the clathrin accessory proteins. However, it should also be noted that designation into one class does

not exclude membership in another. For example, clathrin adaptors may also function as assembly proteins, as is the case for epsin and the AP complexes, which are discussed in the following section.

The delineation of clathrin accessory proteins into different subgroups is needed for clarification of nomenclature since, up until recently, research has focused primarily on the relationship between clathrin and individual proteins. However, the contribution and interaction between clathrin associated proteins themselves must not be overlooked. Many questions regarding protein-protein interactions during clathrin-mediated events remain unanswered. For example, what is the extent of interaction between clathrin adaptors? What is the nature of the relationship between clathrin adaptors and other accessory proteins? Do individual accessory proteins influence the localization of other accessory proteins to endocytic sites? Do clathrin accessory proteins regulate the function of other clathrin associated proteins? Do clathrin accessory proteins have additional roles in cellular processes unrelated to clathrin-mediated endocytosis? The currents study aims to address these questions through characterization of a recently identified *Dictyostelium* clathrin adaptor, Hip1r.

1.3.3 The Role of Accessory Proteins in Clathrin-Mediated Events

To date, more than 20 different known factors have been shown to interact with clathrin structures (Kirchhausen, 2000; Owen et al., 2004). The AP complexes are one of the most well-known of the clathrin adaptor proteins. The best characterized of the 4 classes of AP complexes is AP-2. The AP-2 complex localizes to specific regions of the

plasma membrane and has been shown to bind the cytoplasmic tails of cargo to be internalized (Owen and Evans, 1998; Bonifacino and Traub, 2003). AP-2 promotes clathrin assembly by recruiting clathrin and other accessory factors to the site of endocytosis as well as acting to cluster cargo into the coated pit (Owen et al., 2004; Ahle and Ungewickell, 1989).

AP180/CALM is another clathrin adaptor that influences the assembly of clathrin-coated vesicles. Similar to the AP-2 complex, AP180 recruits clathrin to endocytic sites on the membrane and promotes the formation of clathrin structures (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Ye and Lafer, 1995; Ford et al., 2002). Orthologs of AP180 have been demonstrated to regulate the size and composition of synaptic vesicles as well as other subcellular organelles (Zhang et al., 1998; Nonet et al., 1999; Stavrou and O'Halloran, 2006). AP180/CALM associates with cellular membranes by means of its membrane-binding ANTH (AP180 NH_2-Terminal Homology) domain. The ANTH domain binds regions rich in the phospholipid phosphotidylinositol-4,5-bisphosphate (PIP_2) and is present in other cellular proteins associated with membrane trafficking events (Legendre-Guillemin et al., 2004; Itoh and De Camilli, 2006). The ANTH domain is very similar to another membrane binding motif, the ENTH domain (Legendre-Guillemin et al.., 2004; Itoh and De Camilli; 2006).

The ENTH (Epsin NH_2-Terminal Homology) domain is the most highly conserved region among proteins belonging to the epsin family, members of which are proposed to function as clathrin adaptors for ubiquitinated cargo (Kay et al., 1999; Chen et al., 1998; Wendland, 2002). Like AP-2 and AP180, epsin is thought to recruit clathrin to the plasma membrane (Ford et al, 2001; Kweon et al., 2006). In addition to recruitment, epsin plays a crucial role in the formation of the clathrin-coated pit; the ENTH domain binds phosphotidyl inositols (PIP_2), and by inserting a portion of itself

into the membrane, epsin induces curvature during clathrin polymerization (Ford et al., 2002). In addition to the ENTH domain, the epsin proteins contain ubiquitin-interacting motifs (UIM) and have binding domains for clathrin, AP-2 and EH (Eps-15 Homology) domain-containing proteins along their length (Chen et al., 1998; Kay et al., 1999; Wendland, 2002). In mammalian cells, epsin 1 is associated with clathrin-coated pits and disrupting its interactions with other accessory proteins inhibits endocytosis (Chen et al., 1998). In *Drosophila*, the epsin ortholog, Liquid facets, is necessary for endocytosis of ligands for the Notch receptor and is a substrate for the deubiquitylating enzyme, Fat facets (Cavidad et al., 2000; Overstreet et al., 2003). Studies in yeast have shown mutations in the ENTH domain lead to temperature-sensitive growth and endocytic defects (Wendland et al., 1999). Deletion of both yeast homologs, Ent1 and Ent2, results in cell death however; the ENTH domain of Ent1 is able to rescue the lethality (Wendland et al., 1999). Interestingly, the ENTH domain of yeast was recently shown to influence cytoskeletal organization by interacting with regulators for Cdc42, a key regulator of the actin cytoskeleton (Aguilar et al., 2006).

1.3.4 F-actin and Clathrin-Mediated Endocytosis

In both yeast and mammalian systems, the actin cytoskeleton plays an important role during clathrin-dependent internalization. In yeast, actin polymerization is required for endocytic events (Engqvist-Goldstein and Drubin, 2003). Cortical actin patches, one of three main filamentous actin structures in budding yeast, are thought to be active sites of endocytosis (Moseley and Goode, 2006). More than 30 proteins, many of which have been postulated to act during endocytosis, have been described as components of cortical actin patches (Engqvist-Goldstein and Drubin, 2003). In mammalian cells, loss of functional F-actin only partially inhibits endocytosis but nonetheless, the actin

cytoskeleton has a key role in clathrin-mediated events in these organisms (Yarar et al., 2005; Smythe and Ayscough, 2006).

The current model for actin involvement in endocytosis proposes that actin is recruited to endocytic sites during the later stages of vesicle formation. Actin polymerization assists in vesicle scission, release from the membrane and movement of the clathrin-coated vesicle into the cytoplasm (Kaksonen et al., 2003; Merrifeld et al., 2002; Merrifeld, 2004). While the number of factors shown to interact with or influence F-actin and participate in endocytic events continues to grow, only one family of proteins--the Sla2/Hip1 family--has been demonstrated to interact physically with both clathrin and F-actin (Yang et al., 1999; Henry et al., 2002; Engqvist-Goldstein et al., 1999).

1.3.5 The Sla2/Hip1 Family of Proteins

The Sla2/Hip1 family--which is comprised of the yeast Sla2p protein, its mammalian orthologs Hip1 and Hip1r, a nematode ortholog (ZK370.3) and the recently identified *Dictyostelium discoideum* homolog, Hip1r--share three distinct domains (Yang et al., 1999; Engqvist-Goldstein et al., 1999; Kalchman et al., 1997; Repass et al., submitted). The NH_2-terminus contains an ANTH domain, the membrane-binding motif also present in another clathrin associated protein, AP180 (Ford et al., 2001; Legendre-Guillemin et al., 2004). Adjacent to the ANTH domain, the central region displays a varying number of predicted coiled-coil domains between family members. Finally, the COOH-terminal portion of the protein has a talin-like, F-actin binding domain or THATCH (Talin-Hip1/R/Sla2p Actin-Tethering C-Terminal Homology) domain (Yang

et al., 1999; Engqvist-Goldstein et al., 1999; Kalchman et al., 1997; Brett et al., 2005; Repass et al., submitted).

The founding member of the Sla2/Hip1 protein family, Sla2p (Synthetic Lethal with Abp1), was one of the first mutants identified to be deficient in endocytosis in *Saccharomyces cerevisiae* (Raths et al, 1993). Analysis of Sla2p has shown that the NH_2-terminal domain of Sla2p, which includes the ANTH membrane-binding motif, is essential for growth and endocytosis (Yang et al., 1999; Wesp et al., 1997). The central coiled-coil domain of the protein interacts with clathrin light chain and deletion of clathrin affects the cortical localization of Sla2p (Henry et al., 2002; Newpher and Lemmon, 2006). At the COOH-terminus, the THATCH domain of Sla2p is involved in endocytic events *in vivo* and interacts genetically with the yeast orthologs of epsin, a known clathrin adaptor (Baggett et al., 2003). These studies suggest that Sla2p provides an important link between clathrin and actin during yeast endocytosis, and that Sla2p may function with other clathrin associated proteins to mediate processes occurring at the plasma membrane.

Hip1 (Huntingtin Interacting Protein 1) is predominately expressed in neuronal tissue and was identified in a yeast two-hybrid screen for huntingtin protein binding partners (Kalchman et al., 1997; Wanker et al., 1997). Hip1 contains motifs for binding clathrin and AP-2 (Metzler et al., 2001). Hip1 was demonstrated to bind directly to clathrin light chain and promotes clathrin assembly *in vitro* (Legendre-Guillemin et al., 2002).

The mammalian homolog of Sla2p, Hip1r (Huntingtin Interacting Protein 1 Related), colocalizes with clathrin, the AP-2 complex and early endocytic compartments

(Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001). *In vitro* assays have shown that mammalian Hip1r binds clathrin light chain directly through its central coiled-coil domain and that mHip1r physically links F-actin to clathrin cages (Engqvist-Goldstein et al., 2001; Chen and Brodsky, 2005). Through its central coiled- coil domain, Hip1r is also able to form homodimers or heterodimerize with Hip1 (Legendre-Guillemin et al., 2002). In addition, RNAi-mediated silencing suggests that mHip1r acts to stabilize components of the endocytic machinery at the plasma membrane (Engqvist-Goldstein et al., 2004). Recent work has also demonstrated mHip1r involvement in membrane trafficking events at the trans-Golgi network (Carreno et al., 2004). Taken together, these findings suggest, that the Sla2/Hip1 family of clathrin adaptors act as a physical links between clathrin and actin during membrane trafficking processes in eukaryotic cells.

1.4 DICTYOSTELIUM DISCOIDEUM

1.4.1 *Dictyostelium discoideum* as a Model System

The social soil amoeba, *Dictyostelium discoideum*, is an excellent model system in which to examine a variety of cellular processes. *Dictyostelium* has six chromosomes and approximately 10,000 genes (Cox et al., 1990; Loomis et al., 1995). In nature, the amoeba is a resident of the forest floor and when bacteria are readily available for consumption, exists as a single-celled organism. In this state, the individual cells are haploid, making genetic manipulation relatively easy. Moreover, endocytosis and the regulation of membrane trafficking events are critically important in *D. discoideum* as the

amoeba must constantly adjust its internal environment to keep pace with changes in its external surroundings.

When the supply of nutrients becomes limited, *Dictyostelium* will initiate a developmental program that results in the production of spores (Kessin, 2001; West, 2003). Under starvation conditions, individual amoeba will secrete cyclic AMP (cAMP) as a signal for chemotaxis and aggregation into a gathering of approximately 1 x 10^5 cells over a 24 hour period (Firtel and Chung, 2000; Kessin, 2001). Roughly 80% of the cells in one aggregate are destined to become dormant amoeba, whereas the other 20% will serve as the framework for a multicellular structure, the fruiting body. The fruiting body supports and houses environmentally resistant spores, dormant amoeba encased in a thick, trilaminar spore coat (West and Erdos, 1990; West, 2003). When conditions are once again favorable, the spores will germinate to yield a single amoeba to begin the process again. The developmental program of *Dictyostelium* offers researchers an opportunity to study, among other cellular events, cell differentiation, cell migration and cell-to-cell communication.

1.4.2 Clathrin-Mediated Events in *Dictyostelium*

The *Dictyostelium* genome has one gene for clathrin heavy chain and one gene for clathrin light chain (O'Halloran and Anderson, 1992; Wang et al., 2003). Characterization of the intracellular roles of clathrin has demonstrated the importance of clathrin-dependent processes in the amoeba. In the absence of clathrin heavy chain, cells are unable to complete cytokinesis in suspension and moreover, clathrin heavy chain is essential for the assembly of a functional contractile ring (Niswonger and O'Halloran, 1997a). In growing cells, the absence of clathrin heavy chain results in deficient pinocytosis and osmoregulation (O'Halloran and Anderson, 1992). Furthermore, lack of

heavy chain expression impairs fluid phase uptake as well as the sorting and secretion of lysosomal enzymes (Ruscetti et al., 1994). Deletion of clathrin light chain in growing cells also affects cytokinesis, osmoregulation and decreases the association of heavy chain with the plasma membrane in *Dictyostelium* (Wang et al., 2003). In developing cells, clathrin is required for the production of mature fruiting bodies containing viable spores. Cells lacking functional clathrin exhibited a delay in aggregation and were unable to generate spores (Niswonger and O'Halloran, 1997b). Together, these data demonstrate the importance of clathrin-mediated events in *Dictyostelium*.

1.4.3 Clathrin Accessory Proteins in *Dictyostelium*

Although clathrin-mediated endocytosis in the amoeba closely resembles that of higher eukaryotes, relatively little is known about *Dictyostelium* clathrin associated proteins. To date, only three clathrin adaptor proteins have been described in the literature (Lefkir et al., 2003; Lefkir et al., 2004; Charette et al., 2006; Stavrou and O'Halloran, 2006). The *Dictyostelium* AP-1 complex is required for lysosomal sorting and contractile vacuole biogenesis in addition to phagocytosis and macropinocytosis (Lefkir et al., 2003; Lefkir et al., 2004). The AP-3 complex functions in the endosomal pathway of the amoeba (Charette et al., 2006). In addition, the *Dictyostelium* ortholog of AP180/CALM associates with clathrin at the plasma membrane and influences morphology of the contractile vacuole (Stavrou and O'Halloran, 2006).

The identification and characterization of additional clathrin accessory and adaptor proteins is vital to understanding clathrin-mediated endocytosis in *Dictyostelium* as well as other eukaryotes. For example, which adaptors serve to tether clathrin to the plasma membrane? Are there proteins that act as linkages between clathrin and F-actin during endocytic events? How do clathrin accessory proteins interact with each other to

accomplish endocytosis? Do clathrin associated proteins have additional roles in cellular processes, such as development? Information gleaned will contribute to our knowledge of how eukaryotic cells regulate their internal processes to participate in numerous membrane trafficking events. In this study, I describe a functional analysis of *Dictyostelium* Hip1r, a clathrin adaptor that functions in both growth and development. In addition, I report a newly identified association between two clathrin adaptors, Hip1r and epsin.

Chapter 2

Dictyostelium Hip1r Contributes to Spore Shape and Requires Epsin for Phosphorylation and Localization

2.1 INTRODUCTION

The regulation of membrane trafficking events is vital to the proper functioning of living organisms. Clathrin-mediated endocytosis, the trafficking of coated vesicles from the plasma membrane, occurs in all eukaryotes and is thought to involve complex interactions between a multitude of factors and the plasma membrane (Brodsky et al., 2001; Lafer, 2002; Perrais and Merrifeld, 2005). In particular, a host of proteins are thought to function as clathrin adaptors and accessory proteins that associate with clathrin triskelions to tether and assemble the triskelions into an organized lattice on the plasma membrane (Owen et al., 2004; Sorkin, 2004). Each of the many clathrin-associated proteins are generally conserved in structure and amino acid sequence in most eukaryotic cells, suggesting that an individual clathrin associated protein contributes common features to the assembly or function of clathrin lattices. Nonetheless, many of the interactions between the adaptors and/or accessory proteins and their specific contributions to cellular function remain uncharacterized.

One family of clathrin adaptors--the Sla2/Hip1 family--has distinct domains demonstrated to interact both with clathrin and with the actin cytoskeleton (Chen and Brodsky, 2005; Legendre-Guillemin et al., 2002; Qualman and Kessels, 2002). At the NH_2-terminus, members of this family contain an AP180 NH_2-terminal homology domain (ANTH), a domain found in proteins predicted to function in trafficking events

via interactions with phosphotidyl inositols in membranes (Legendre-Guillemin et al., 2004; De Camilli et al., 2002; Itoh et al., 2001). Adjacent to the NH_2-terminal region, a predicted coiled-coil region of variable length is postulated to mediate protein-protein interactions, including binding clathrin (Wesp et al., 1997; Engqvist-Goldstein et al., 1999; Mishra et al., 2001; Henry et al., 2002). Finally, the COOH-terminal region of the Sla2/Hip1 orthologs contains a talin-like, F-actin binding domain, also called the THATCH domain that has been demonstrated to interact with F-actin (Brett et al., 2005; McCann and Craig, 1997; Yang et al., 1999; Engqvist-Goldstein et al., 1999 and 2001). The domain structure of Sla2/Hip1 family members suggests that they serve as molecular links between the plasma membrane, cortical actin and the clathrin endocytic machinery (Henry et al., 2002; Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2002).

The founding member of the Sla2/Hip1 protein family, Sla2p (Synthetic Lethal with Abp1), is essential for endocytic events in *Saccharomyces cerevisiae* (Raths et al, 1993). The central coiled-cold domain of Sla2p binds clathrin light chain and the absence of clathrin affects the cortical localization of Sla2p (Henry et al., 2002; Newpher and Lemmon, 2006). The THATCH domain of Sla2p is involved in endocytic events *in vivo* and interacts genetically with the yeast orthologs of epsin, a protein found in a wide variety of eukaryotic organisms that acts as an important adaptor for clathrin on the plasma membrane (Baggett et al., 2003).

Like the Sla2p/Hip1 proteins, epsin has a membrane-binding domain at its NH_2-terminus, called an ENTH domain, which similarly binds the phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) (Itoh et al., 2001). However, unlike the

ANTH domain of Hip1r, the epsin ENTH domain inserts into the membrane when bound to PIP_2 (Ford et al., 2002; Stahelin et al., 2003; Kweon et al., 2006). Along its COOH-terminal domain, epsin contains multiple binding sites for clathrin and other clathrin accessory proteins (Chen et al., 1998; Kay et al., 1999). Thus, epsin is thought to bind to the coated pit and induce the membrane invagination necessary for vesicle bud formation (Ford et al., 2002; Hurley, 2006).

The social soil amoeba, *Dictyostelium discoideum*, is an excellent model system for the study of cellular trafficking events in both growing and developing cells as membrane budding and fusion is of critical importance in the organism (Kessin, 2001; Maniak, 2003). Similarly to other eukaryotes, *Dictyostelium* contains clathrin-coated vesicles that bud from the plasma membrane as well as intracellular organelles (O'Halloran and Anderson, 1992; Damer and O'Halloran, 2000; Wang et al., 2006). *Dictyostelium* cells have clathrin adaptors common to those found in other eukaryotic cells (Stavrou and O'Halloran, 2006; Charrette et al., 2006; Lefkir et al, 2003). When nutrients are depleted, *Dictyostelium* cells can be induced to develop into multicellular structures, offering a model system to investigate clathrin and adaptor function during both growth and during development.

Here I have identified Hip1r, a *Dictyostelium* member of the Sla2p/Hip1 family. Similar to members of this family in other species, *Dictyostelium* Hip1r localizes into punctae on the plasma membrane that associate with clathrin and other associated proteins. I identified a novel requirement for epsin for both the phosphorylation and the restricted localization of Hip1r into punctae to the plasma membrane, a role that can be supplied by the membrane-binding ENTH domain of epsin. I also uncovered an essential

role for Hip1r in development and formation of ovoid and robust spores during *Dictyostelium* development.

2.2 RESULTS

2.2.1 Identification of *Dictyostelium* Hip1r, a New Member of the Sla2/Hip1 Family of Proteins

To identify potential orthologs of the Sla2/Hip1 family of proteins, I searched the *Dictyostelium* Gene Database (Chisholm et. al., 2006; www.dictybase.org) and found one gene (DDB0232318), located on chromosome 4, that I named *hipA.* The gene encodes a single protein of 961 amino acids with a predicted molecular weight of 107 kilodaltons. Analysis of the predicted reading frame revealed a gene product that shared domains with members of the Sla2/Hip1 family of proteins. At its NH_2-terminus (amino acids 1-125), the *Dictyostelium* Hip1r homolog contains an ANTH domain that shared approximately 32% amino acid identity with the other family members. The COOH-terminal portion of the protein (amino acids 758-959) had an talin-like domain, or THATCH domain that shared 34% identity with other members of the Sla2/Hip family of proteins. Additionally, the central portion of the protein contained regions predicted to be coiled-coil structures. Across its entire length, Hip1r shares approximately 24% identity with Sla2p of *Saccharomyces cerevisae* and 20% and 19% identity with mouse Hip1r and human Hip1r, respectively. Hip1r has a central proline rich region (amino acids 247-294) and, similar to yeast Sla2p, a glutamine rich (amino acids 312-401) region absent from the mammalian orthologs (Fig. 2.1).

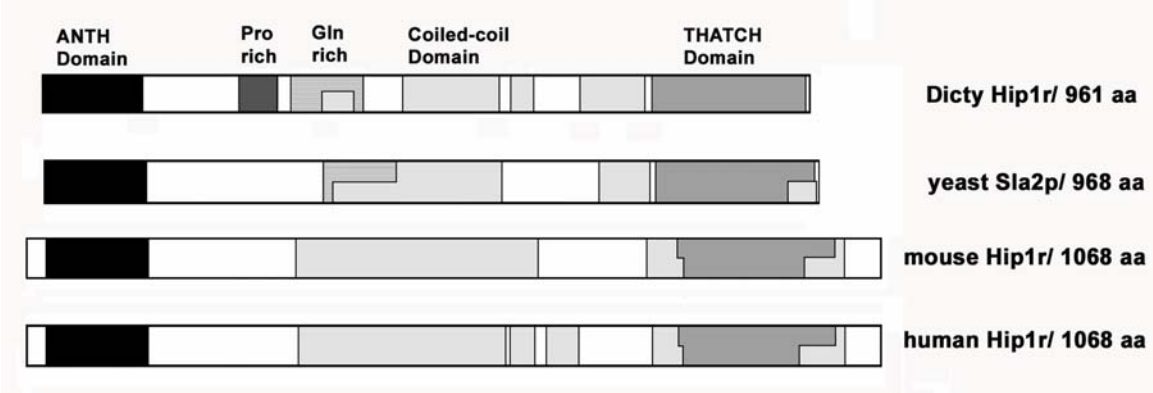

Figure 2.1 Domain structures of Hip1r orthologs in different species. Like other Sla2/Hip1 family members, Hip1r contains an ANTH domain, a central coiled-coil region and THATCH domain as well as proline and glutamine-rich regions. Over its entire length, Hip1r shares 24% identity with Sla2p and 20%/19% with mouse and human Hip1r, respectively. Overlap of shaded regions indicates areas of overlap between domains Dicty: *Dictyostelium discoideum*; Yeast: *Saccharomyces cerevisiae*; Mouse: *Mus Musculus*; Pro: Proline; Gln: Glutamine.

2.2.2 Hip1r is a Soluble, Phosphorylated Protein that Associates with Membranes

To gain insight into the function of Hip1r, I examined potential interactions of the protein with membranes and/or cortical structures. To test for a possible association with membranes, lysates of wild type cells were analyzed by differential centrifugation (Fig. 2.2A). Immunoblots stained with anti-Hip1r antibodies demonstrated that most of the cellular pool of Hip1r sedimented with large plasma membrane fragments and/or large membrane bound organelles (LSP) while the remaining population of the protein remained soluble (LSS, HSS).

In addition, I examined the association of Hip1r with Triton X-100 extracted cytoskeletal fractions. When cell lysates were extracted with the non-ionic detergent TX-100, a portion of Hip1r fractionated into the insoluble pellet, suggesting an association with cytoskeletal factors (Fig. 2.2B). Extraction of the TX-100 insoluble fraction with high salt shifted Hip1r into the soluble fraction, suggesting an ionic interaction with cytoskeletal elements (Fig. 2.2B).

Interestingly, immunoblots probed with anti-Hip1r antibodies revealed two Hip1r species. To determine if this was due to phosphorylation of the Hip1r protein, cell lysates were incubated with calf intestinal phosphatase (Fig. 2.2C). Okadaic acid, an inhibitor of serine/threonine phosphatases, was included in control samples. After 25 minutes of incubation of the lysate with the phosphatase, the slower migrating band of the doublet disappeared from the test sample but remained in the control sample, indicating that Hip1r is phosphorylated and is most likely modified on a serine or threonine residue.

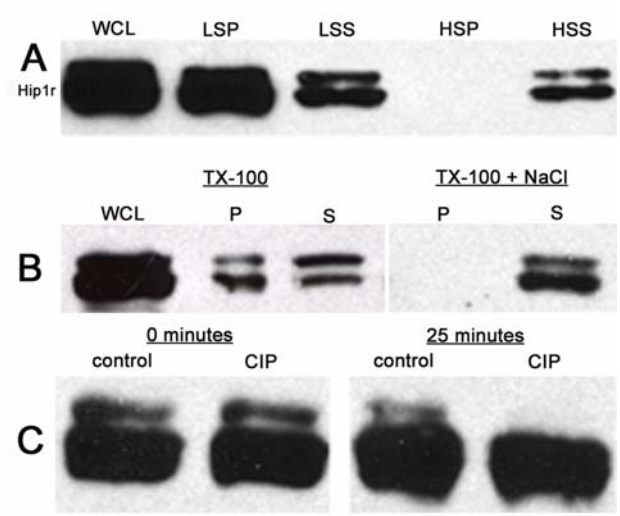

Figure 2.2 Hip1r is a phosphorylated protein that associates with membrane and Triton-X100 insoluble fractions. (A) Subcellular cell fractionation of wild type cells. Most of the cellular pool of Hip1r associates with large membrane structures (LSP) while the rest is soluble (HSS). (B) Triton-X fractionation of Hip1r. Cells were resuspended into buffer containing 0.5% Triton-X100 and fractionated, resulting in a portion of Hip1r sedimenting with the insoluble fraction (P). Extraction of the detergent-insoluble pellet with high salt buffer shifts Hip1r to the soluble fraction (S). (C) Hip1r is phosphorylated *in vivo*. Cells were lysed and incubated with Calf Intestinal Phosphatase (CIP) for 25 minutes resulting in the disappearance of the upper band of the Hip1r doublet. Control lane includes CIP plus Okadaic Acid, a phosphatase inhibitor.

WCL: Whole Cell Lysate; LSP: Low Speed Pellet; Pellet; LSS: Low Speed Supernatant; HSP: High Speed Pellet; HSS: High Speed Supernatant; S: Supernatant; P: Pellet CIP: Calf Intestinal Phosphatase-treated sample; control: CIP+ Okadaic Acid

2.2.3 Hip1r Localizes within Punctae on the Plasma Membrane

The fractionation pattern of Hip1r suggested that Hip1r is a soluble protein associated with membranes and the cytoskeleton. I also examined its subcellular localization. Wild type cells were immunostained with affinity-purified anti-Hip1r antibodies and examined by fluorescence microscopy. While some lightly stained punctae were found in the cytoplasm, the brightest fluorescence was associated with discrete punctae on the plasma membrane (Fig. 2.3A-B). Unlike its mammalian ortholog, Hip1r did not exhibit a juxtanuclear staining pattern. Focusing on the surface of cells expressing GFP-labeled clathrin and immunostained with Hip1r antibodies revealed that Hip1r colocalized with clathrin structures, although not all Hip1r punctae were associated with clathrin (Fig. 2.3C-E). While both clathrin and Hip1r punctae were present on the plasma membrane, less than half of the Hip1r punctae overlapped with clathrin.

The staining pattern of Hip1r was similar to the pattern of the *Dictyostelium* epsin ortholog which also localizes within clathrin-associated punctae on the plasma membrane (Brady and O'Halloran, unpublished). Similar in structure to epsins from other species, the single *Dictyostelium* epsin contains an NH_2-terminal ENTH (Epsin NH_2-terminal Homology) domain and motifs for binding clathrin, AP2, and proteins with EH domains (Brady and O'Halloran, unpublished). To examine the relative localization of Hip1r and epsin, cells expressing GFP-labeled epsin were fixed, immunostained with affinity-purified anti-Hip1r antibodies and examined by fluorescence microscopy. Hip1r and epsin colocalized extensively within punctae along the plasma membrane (Fig. 2.3F-H).

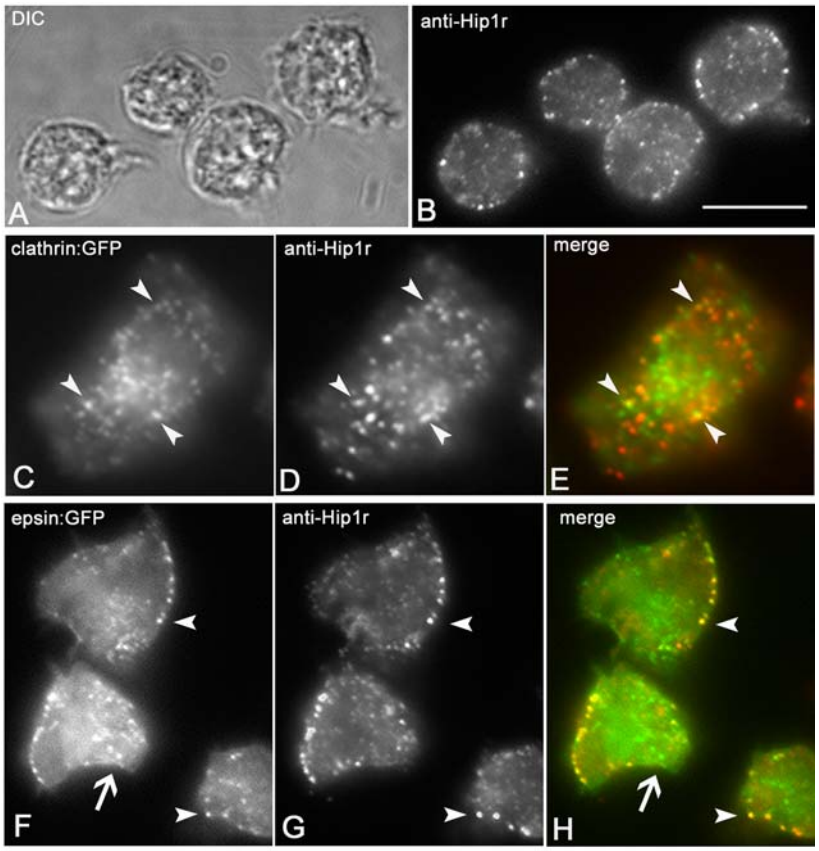

Figure 2.3 Hip1r localizes to the plasma membrane and cytoplasm and displays a functional interaction with epsin. Growing cells were fixed and stained with anti-Hip1r. (A) Cells were viewed with differential interference contrast microscopy (DIC). (B) Cells stained with anti-Hip1r show abundant Hip1r punctae at the cell cortex when viewed with fluorescence microscopy. (C-E) Focusing on the surface of cells expressing clathrin:GFP construct (clathrin:GFP) shows partial colocalization of clathrin (green) with Hip1r (anti-Hip1r, red) as indicated by white arrows in merged image (E). (F-H) Cells expressing an epsin:GFP fusion protein (epsin:GFP, green) and stained with anti-Hip1r antibodies (anti-Hip1r, red) exhibit extensive colocalization at the plasma membrane (arrowheads). Occasionally, punctae contain epsin but not Hip1r (arrow) Scale bar, 10μm.

2.2.4 Hip1r Exhibits a Potential Functional Interaction with Epsin

The extensive co-localization of Hip1r and epsin within cell surface punctae (Fig. 2.3F-H) prompted us explore to the relationship between the two proteins. To test whether Hip1r influenced the localization of epsin within punctae on the plasma membrane, I examined the distribution of epsin in Hip1r null cells. Hip1r null cells were generated by using homologous recombination to delete the *hipA* gene (Fig. 2.4). Inspection by fluorescence microscopy of Hip1r null cells transformed with an epsin:GFP fusion protein showed a distribution of epsin that was similar to that observed in wild type cells (Fig 2.5A). In both strains, epsin-labeled punctae were at the plasma membrane almost exclusively (Fig. 2.5A-B).

To test the reciprocal possibility, that epsin influenced Hip1r localization; I examined the distribution of Hip1r in epsin mutant cells. Epsin null cells immunostained with anti-Hip1r serum revealed a mislocalization of the Hip1r protein (Fig. 2.5D). In contrast to wild type cells (Fig. 2.5C), Hip1r was not concentrated at the surface of epsin null cells. Rather, the epsin null cells displayed an increased number of Hip1r-labeled punctae structures within the cytoplasm with few Hip1r-labeled punctae at the cell surface. This suggested that epsin could play a role either recruiting or stabilizing Hip1r within punctae on the plasma membrane.

The domain structure of the epsin protein includes an NH_2-terminal membrane-binding ENTH domain and a COOH-terminal domain that contains multiple motifs for binding clathrin and clathrin accessory proteins. To determine whether expression of these domains could restore the proper localization of Hip1r, I examined epsin null

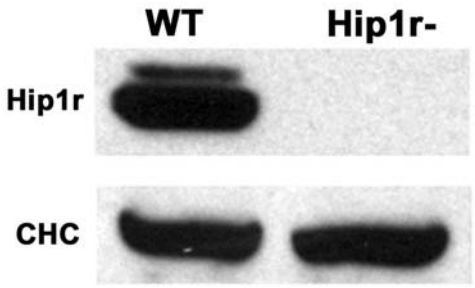

Figure 2.4 The absence of Hip1r expression in Hip1r null cells was confirmed by immunoblotting whole cell lysates of wild type cells (WT) and Hip1r mutant cells (Hip1r-) with anti-Hip1r polyclonal serum. Equal loading was confirmed by immunoblotting with anti-clathrin heavy chain polyclonal serum (CHC).

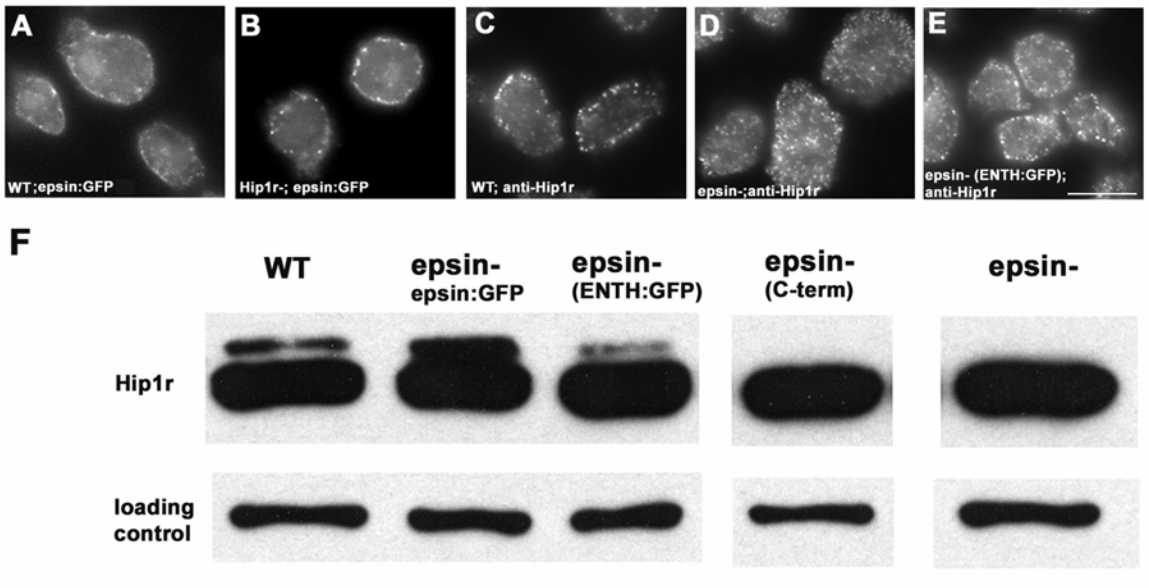

Figure 2.5 Hip1r phosphorylation and membrane localization is dependent upon the NH_2-terminal ENTH domain of epsin. (A-B) In wild type (A, WT) and Hip1r null cells (B, Hip1r-), epsin:GFP (epsin:GFP) exhibits localization to the plasma membrane. (C-D) In contrast to wild type cells (C, WT), Hip1r punctae (anti-Hip1r) are not restricted to the cell periphery in epsin null cells (D, epsin-). (E) Expression of the epsin ENTH domain [epsin- (ENTH:GFP)] partially restores the localization of Hip1r to the periphery. Scale bar, 10μm. (D) Epsin influences the phosphorylation state of Hip1r. Immunoblot of whole cell lysates of epsin null cells expressing epsin:GFP (epsin-, epsin:GFP), the ENTH domain of epsin alone (epsin-, ENTH:GFP), and wild type cells (WT) probed with anti-Hip1r exhibit double bands, indicative of Hip1r phosphorylation. In epsin null cells (epsin-) and cells expressing only the epsin C-terminus (epsin-, C-term) Hip1r is not phosphorylated. Equal loading of lysate was confirmed by the presence of an unrelated background band recognized by the polyclonal serum.

cells that expressed the ENTH domain. When expressed in epsin null cells, an NH_2-terminal construct that included the ENTH domain was sufficient to restore the restricted localization of Hip1r into peripheral punctae in approximately half (47%) of the cells examined (Fig. 2.5E).

The possibility that epsin influenced the phosphorylation the Hip1r protein was also examined. Lysates of wild type, epsin null cells, and epsin null cells expressing epsin:GFP were analyzed in immunoblots stained with anti-Hip1r serum. While the anti-Hip1r antibodies recognized two bands in the wild type and epsin null cells expressing epsin:GFP, only a single band was stained in the epsin null cells (Fig. 2.5F). These results suggest that epsin is essential for the localization of Hip1r to the plasma membrane as well as its phosphorylation state. Moreover, expression of the NH_2-terminal ENTH domain of epsin null cells also restores phosphorylation of Hip1r (Fig 2.5F). In contrast, cells that expressed the COOH-terminal domain contained only the non-phosphorylated species of Hip1r (Fig. 2.5F).

2.2.5 The Absence of Hip1r Expression Does Not Affect Clathrin Localization or Cortical F-actin Organization

Because members of the Sla2/Hip1 family of proteins have been shown to participate in membrane trafficking events, I examined the Hip1r null cells for phenotypes previously shown to be defective in cells lacking clathrin (O'Halloran and Anderson, 1992; Ruscetti et al., 1994; Niswonger and O'Halloran, 1997a and 1997b; Wang et al., 2003). To test for a possible defect in cytokinesis, Hip1r mutant cells were

assessed for growth in suspension. Unlike clathrin mutants, Hip1r null cells were able to complete cytokinesis in suspension (Fig. 2.6A). Likewise, Hip1r mutant cells were not defective in pinocytosis as measured by the internalization of the fluid phase marker, FITC-dextran (Fig. 2.6B). Once internalized, the marker recycled to the extracellular milieu with kinetics similar to that of wild type cells (Fig. 2.6C).

Disruption of mammalian Hip1r or yeast Sla2p expression has been shown to result in clathrin mislocalization as well as aberrant actin phenotypes (Wesp et al., 1997; Yang et al., 1999; Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2004). To determine whether loss of Hip1r expression affected clathrin localization, Hip1r null cells expressing clathrin:GFP constructs were examined using fluorescence microscopy. Wild type and Hip1r null cells showed similar numbers and intensities of clathrin punctae on their plasma membranes (Fig. 2.7A-B). To test for actin abnormalities, I stained the actin cortex of wild type and Hip1r null cells with fluorescently-labeled phalloidin and examined them by fluorescence microscopy. Comparison of actin structures in wild type and Hip1r null cells showed no apparent differences between wild type and mutant cells. Both cell lines demonstrated a thick band of F-actin at the cell cortex, suggesting that loss of Hip1r expression does not affect the overall organization of cortical actin (Fig. 2.7C-D).

2.2.6 Hip1r Mutant Cells Develop into Fruiting Bodies

When nutrients are depleted from the surrounding medium, *Dictyostelium* cells initiate a developmental program whereby approximately 1 x 10^5 starving cells aggregate

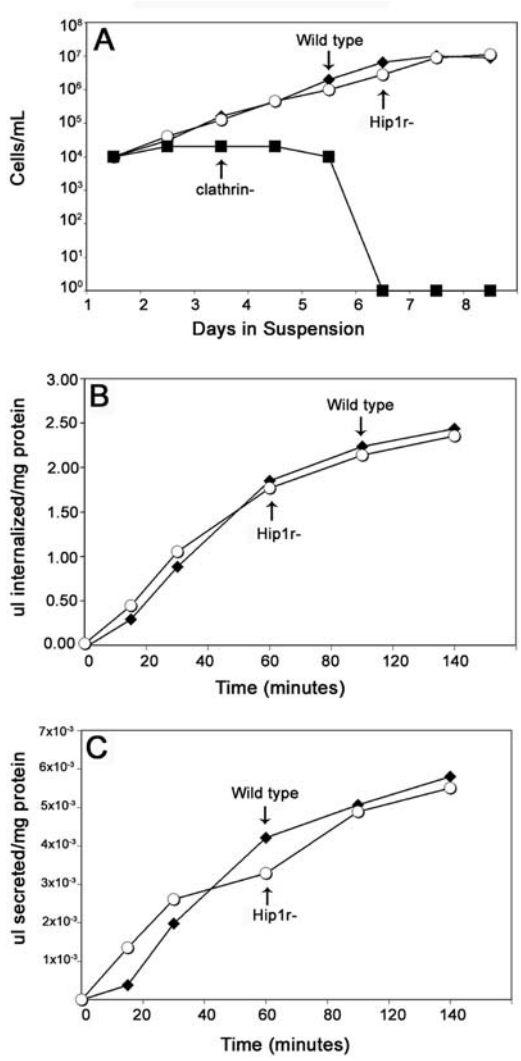

Figure 2.6 Growing Hip1r null cells complete cytokinesis and are not deficient in pinocytosis or secretion. (A) Hip1r null cells are able to complete cytokinesis as measured by growth in suspension cultures. Wild type (closed diamonds) and Hip1r null cells (open circles) were grown in suspension. Clathrin null cells (closed squares), which are deficient in cytokinesis in suspension cultures, were included as a control. (B) Hip1r null cells are not deficient in pinocytosis. Wild type (closed diamonds) and Hip1r null cells (open circles) were incubated with FITC-labeled dextran [2 mg/ml]. At the indicated time points, samples were collected and the amount of internalized FITC-labeled dextran was determined with a fluorometer. (C) Hip1r null cells are not deficient in recycling an endocytosed marker. Wild type (closed diamonds) and Hip1r null cells (open circles) were loaded with FITC-labeled dextran [2 mg/ml] for 2 hours to fill their endocytic compartments. Cells were then incubated in media and, at the indicated time points, samples were collected, washed and the amount of internalized FITC-dextran remaining in the cells was determined with a fluorometer.

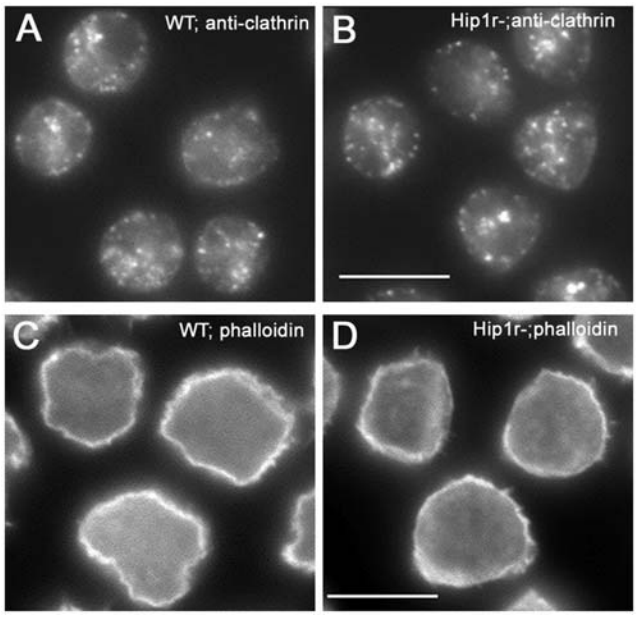

Figure 2.7 Hip1r null cells display normal clathrin and cortical actin. (A-B) Wild type and Hip1r null cells were fixed and stained with anti-clathrin antibodies. Both wild type (WT) and Hip1r null cells (Hip1r-) show fluorescent punctae at the plasma membrane and juxtanuclear staining. (C-D) To visualize F-actin, wild type and Hip1r null cells were stained with rhodamine-conjugated phalloidin. Both wild type (WT) and Hip1r null cells (Hip1r-) show prominent staining of F-actin at the cellular cortex. Scale bar, 10μm

to form the fruiting body, a multicellular structure consisting of a thin stalk supporting a sorus that contains spores (Kessin, 2001). Approximately one-third of the aggregating cells are destined to form the stalk while the remaining cells become spores, dormant amoebae protected by a thick spore coat. When environmental conditions are suitable, these dormant amoeba will emerge from their protective spore coats to produce viable, growing cells. Immunoblots of cells at different stages of development show two bands of the Hip1r protein were expressed throughout all stages of development (data not shown). To test for a possible role in development, wild type and Hip1r null cells were inoculated onto agar plates containing lawns of bacteria. After they depleted the bacteria, Hip1r null cells aggregated without delay, forming fruiting bodies that were indistinguishable from fruiting bodies formed by wild type cells (Fig. 2.8A-B).

To inspect the morphology of the cells within the developmental structure, I examined fruiting bodies on coverslips by Differential Interference Contrast (DIC) microscopy. The stalk cells in the fruiting bodies formed by Hip1r null mutants were similar in size and appearance to stalk cells of wild type fruiting bodies (Fig. 2.8C-D). Both wild type and mutant stalk structures were comprised of vacuolated cells with distinct cell borders.

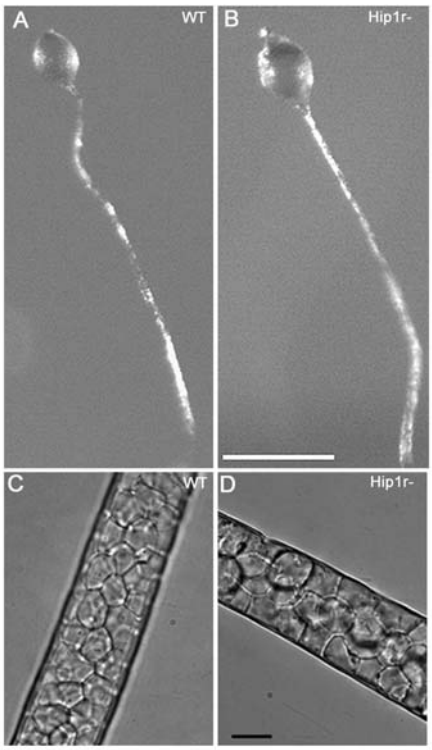

Figure 2.8 Hip1r null mutants develop normally into fruiting bodies. Wild type and Hip1r null cells were harvested and inoculated onto non-nutrient agar plates to induce the *Dictyostelium* developmental program. (A-B) Both wild type (WT) and Hip1r null cells (Hip1r-) produce fruiting bodies consisting of a round sorus, supported by a thin stalk. Scale bar, 0.25 mm (C-D) The stalks from wild type (WT) and Hip1r null cells (Hip1r-) are similar in size and cellular organization. Scale bar, 10μm.

2.2.7 Hip1r Mutant Cells Produce Spores with Abnormal Morphology and Reduced Viability

In contrast with the normal stalk cells within the fruiting body formed by Hip1r null cells, the morphology of Hip1r null spores differed dramatically from wild type spores (Fig. 2.9A, C). Wild type cells produced spores that were elliptical. In contrast, Hip1r mutant spores were round (Fig. 2.9B, D).

A major component of the *Dictyostelium* spore coat is the polysaccharide cellulose. To determine whether a lack of cellulose was associated with the abnormal shape of the Hip1r null spores, I stained wild type and Hip1r mutant spores with calcofluor, a cellulose-binding reagent. The spore coat of Hip1r null cells stained brightly with calcofluor, demonstrating the presence of cellulose within the coat of the mutant spores (Fig. 2.9C-D).

To test the viability of the round, Hip1r mutant spores, I compared germination rates between wild type spores and Hip1r null spores. Spores harvested from sori of the two cell lines were incubated with bacteria on agar plates. The plates were monitored to determine whether viable amoeba emerged from the spores to grow and clear plaques on the bacterial lawn. Spores harvested from the sori of both wild type and mutant fruiting bodies demonstrated comparable survival rates: 70-80% of spores yielded amoeba capable of growing on bacteria (Fig. 2.9E).

In the natural world, the spore coat must protect the dormant amoeba from a variety of environmental onslaughts such as chemical and temperature fluctuations. Wild type spores are encased in a tough, trilaminar spore coat that can withstand harsh

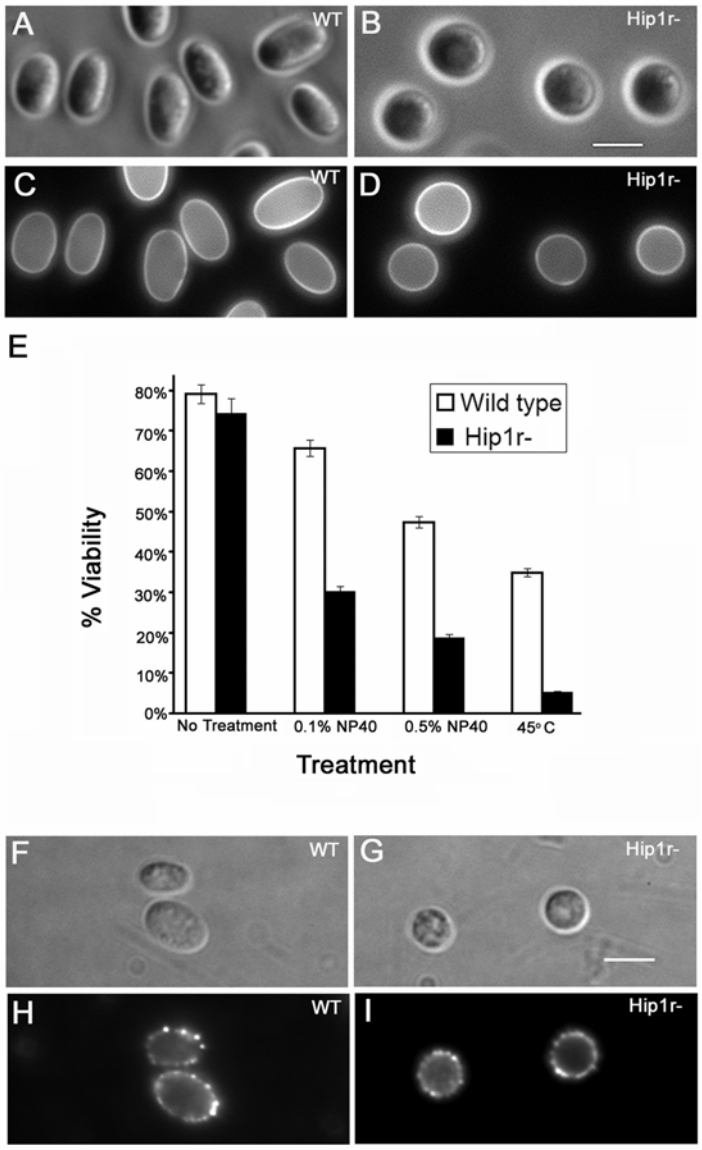

Figure 2.9 *Dictyostelium* Hip1r null cells produce round spores with reduced viability. (A-D) Wild type and Hip1r null spores were harvested, stained with the cellulose-staining dye, calcoflour, and viewed under Differential Interference Contrast (DIC) or fluorescence microscopy. Wild type (WT) spores are elliptical in morphology whereas Hip1r null spores (Hip1r-) are round. Scale bar, 5μm. (E) When treated with detergent or heat, Hip1r null spores (Hip1r-) show decreased viability. Wild type and Hip1r null spores were harvested and left untreated, subjected to washes with the detergent NP40 or heat-treated for 20 minutes. Spores were then plated on a bacterial lawn where they germinated and grew. Cleared plaques were counted as a measure of spore germination. Error bars represent SE. (F-I) Hip1r null cells secrete outer layer spore coat proteins. Wild type (WT) and Hip1r null spores (Hip1r-) were harvested, fixed and stained with an antibody against an outer spore coat protein, SP70, and viewed with DIC (F-G) or fluorescence microscopy (H-I). Scale bar, 5μm.

conditions (Kessin, 2001; West, 2003). To test the resilience of the Hip1r null spore coats, I tested spore viability of wild type and mutant spores after detergent and heat treatments (Fig. 2.9E). After washing spores with the non-ionic detergent NP-40, I plated them on a lawn of bacteria and measured their ability to germinate and grow. The detergent treatment reduced the viability of mutant spores to approximately half that of wild type spores. Heat treatment of spores revealed even more vulnerability in the Hip1r mutant spores. After spores were heated to 45°C for 20 minutes, 35% of amoebae from wild type spores were viable, but only 7% of the null spores yielded viable amoeba.

2.2.8 Hip1r Null Cells are Not Deficient in Pre Spore Vesicle (PSV) Fusion

Because spore coat proteins must be secreted to the extracellular face of the plasma membrane to form a protective coat, I postulated that the secretion of the proteins contained in prespore vesicles might be defective in Hip1r null strains (Srinivasan et al., 2000a; West, 2003). Preassembled spore coat proteins are stored in specialized vesicles called prespore vesicles, or PSVs (Srinivasan et al., 1999, 2000a). These vesicles accumulate within the cytoplasm of the prespore cell until an as-yet-unidentified signal is received. Upon receipt of this signal, the prespore vesicles fuse with the plasma membrane and deposit spore coat proteins on the extracellular surface (Srinivasan et al., 2000a). To determine if prespore vesicle fusion with the plasma membrane was defective in Hip1r mutant cells, I examined wild type and Hip1r null spore coats for the presence of SP70, a spore coat protein contained within the specialized vesicles of prespore cells (Gomer et al., 1986; Fosnaugh et al., 1994). Mature spores from wild type and Hip1r null

fruiting bodies were harvested and immunostained with an antibody directed against SP70. In both wild type and Hip1r null spores, the anti-SP70 antibody labeled the patches that outlined the periphery of the spore without staining the center of the spores (Fig. 2.9F-I) This staining pattern suggested that Hip1r mutant cells successfully deliver spore coat proteins from prespore vesicles to the extracellular surface of spores.

2.2.9 Hip1r Null Spores Have a Disorganized Spore Coat

I used electron microscopy to examine the structure of the spores found in Hip1r null fruiting bodies. As found with light microscopy, scanning electron microscopy also showed oblong wild type spores and round mutant Hip1r spores. In addition, the surfaces of many of the Hip1r spores were wrinkled. When wild type and Hip1r null spores were washed briefly with detergent prior to preparation for scanning electron microscopy, most wild type spores remained oblong, whereas the majority of Hip1r spores collapsed into flattened sacs, suggesting that the mutant spore coat was fragile (Fig. 2.10A-D).

I used transmission electron microscopy to examine the ultra-fine structure of the spore coat. The *Dictyostelium* spore coat is comprised of three layers: an outer protein layer, a middle layer composed of cellulose and an inner protein layer adjacent to the plasma membrane of the dormant amoebae (West, 2003). Images of the wild type spore coat show an outer and inner protein layer with a middle region consisting of material that appeared to be cross-linked or arranged in fibrils. (Fig 2.10E) In contrast with

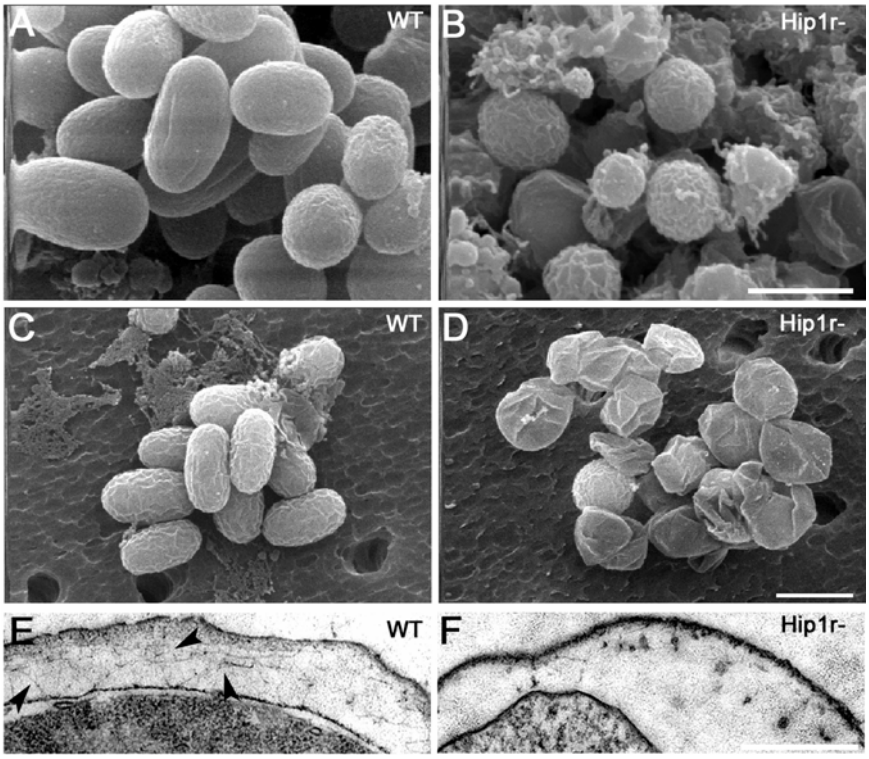

Figure 2.10 Hip1r null spore coats lack organized cellulose. Wild type and Hip1r null spores were collected, fixed and stained (see Experimental Procedures). (A-B) Wild type (WT) and Hip1r null spores (Hip1r-) were collected and washed without detergent. While wild type spores are elliptical, null spores are round. (C-D) Washing wild type spores (WT) and Hip1r null spores (Hip1r-) with 0.1% NP40 before fixation results in the collapse of the mutant spores. Scale bars, 5μm. (E-F) Transmission EM of spores shows a middle layer of protein arranged in fibrils or cross-linked in wild type (WT, arrows), that is absent for the spore coat of mutant spores (Hip1r-). Scale bar, 500 nm.

wild type spore coats, the spore coat formed by mutant Hip1r cells was disorganized and varied in thickness. Moreover, the coat lacked the cross-linked fibrils found in the middle layer of wild type spore coats (Fig. 2.10F).

2.3 DISCUSSION

In this study of *Dictyostelium* Hip1r, I discovered a previously unidentified interaction between Hip1r and another clathrin adaptor, epsin. Hip1r displayed a strong dependence on epsin, for both phosphorylation and the restricted localization of Hip1r into plasma membrane punctae. In addition, I identified a critical role for Hip1r in development. During fruiting body formation, Hip1r was required for the proper organization of the spore coat, which is necessary to form oval spores that were sufficiently robust to withstand harsh conditions.

2.3.1 Potential Roles for Hip1r in *Dictyostelium* Spore Formation

To date, a role for members of the Sla2/Hip1 family of proteins in development has not been identified. The generation of environmentally resistant spores is the end result of *Dictyostelium* development and is important for the organism to survive harsh conditions in nature. The spore coat that encloses dormant amoebae within the spore is comprised of cellulose and glycoproteins (West and Erdos, 1990). Most of these glycoproteins are delivered to the exterior of prespore cells when prespore vesicles, containing pre-assembled protein complexes, fuse with the plasma membrane (Srinivasan

et al., 1999, 2000a). This regulated secretion is a prerequisite for both the synthesis and the organization of cellulose in the spore coat (Srinivasan et al., 2000b; Metcalf et al., 2003). After the spore coat proteins are secreted to the extracellular side, the enzyme responsible for cellulose synthesis, cellulose synthase, is positioned in the plasma membrane and synthesizes cellulose directly into the outer layer of the spore coat (West 2003; Metcalf et al., 2003; Srinivasan et al., 2000b).

Here I show that Hip1r is required for the formation of a normal *Dictyostelium* spore coat. While the Hip1r mutant spore coat stains with calcofluor, demonstrating the presence of cellulose, electron micrographs of the spore coat show an absence of cross-linked fibrils, suggesting that the cellulose may be incorrectly organized to form a structurally sound protective coat. While *Dictyostelium* spores adopt their elliptical morphology prior to cellulose deposition, organized cellulose is required to retain this shape (Zhang et al., 2000; West, 2003). One determinant for organized cellulose fibrils is the glycoprotein complex secreted from prespore vesicles; this complex is known to bind cellulose and to have a polarized position within the spore coat (McGuire and Alexander, 1996; Srinivasan et al., 2000b). Hip1r mutant spores stain with SP70, a major component of this glycoprotein complex, so Hip1r is not required for the overall secretion of prespore vesicle contents. As a scaffolding protein with binding sites for actin, the plasma membrane and a coiled-coil domain, Hip1r could be required for the proper positioning of the glycoprotein complex and/or the cellulose synthase enzyme.

2.3.2 *Dictyostelium* Hip1r and the Sla2p/Hip1 Family

In contrast with orthologs in other species, *Dictyostelium* Hip1r did not associate with the majority of cell surface clathrin punctae nor did Hip1r null mutants exhibit defects in cortical actin structure, clathrin localization or display phenotypes normally associated with clathrin or actin deficiencies. Overall, our results suggest that if Hip1r interacts functionally with clathrin or actin, this function could be redundant with other proteins. Interestingly, analysis of Hip1r amino acid sequence reveals the protein lacks the recently identified amino acid motif necessary for mammalian Hip1 interaction with clathrin light chain (Legendre-Guillemin et al., 2005). On the other hand, the Hip1r ANTH domain does contain four conserved lysine residues shown to be necessary for the interaction of *Saccaromyces cerevisiae* Sla2p with the phospholipid phosphatidylinositol-4,5-bisphosphate (Sun et al., 2005).

2.3.3 Hip1r Interacts with Epsin

Using immunofluorescence and biochemical assays I have shown that Hip1r is a phosphorylated protein that is distributed into punctae associated with the plasma membrane. A genetic interaction between the yeast ortholog of Hip1r, Sla2p and epsin has been reported previously but how these two adaptors influence each other is not known (Baggett et al., 2003). I identified an essential contribution of epsin to both the localization and phosphorylation state of *Dictyostelium* Hip1r. In wild type cells, Hip1r displays an extensive colocalization with epsin on the plasma membrane while in epsin null cells, Hip1r is displaced from the plasma membrane. Furthermore, the

phosphorylated form of Hip1r was absent in epsin null cells. Both the epsin-dependent phosphorylation and plasma membrane localization were rescued by an NH_2-terminal fragment of epsin containing the PIP_2-binding ENTH domain. An interaction between *Dictyostelium* Hip1r and epsin is also supported by the almost identical developmental phenotypes exhibited by *Dictyostelium* Hip1r and epsin null mutants. During development, both Hip1r and epsin null cells produce round, rather that elliptical, spores with reduced viability (this study; Brady and O'Halloran, unpublished).

Based on our results, I propose that epsin recruits and /or positions Hip1r at the plasma membrane of growing and developing *Dictyostelium* cells. The epsin-dependent positioning of Hip1r at the cell cortex could allow Hip1r to be phosphorylated, perhaps by membrane-associated kinases, and thus influence its function. Similarly, an epsin-dependent phosphorylation event could position Hip1r at the membrane of prespore cells where the complex could function to promote the proper organization of determinants for cellulose synthesis during spore formation. I favor the possibility that phosphorylation of Hip1r occurs at the plasma membrane since the NH_2-terminal ENTH domain of epsin, which is able to rescue Hip1r phosphorylation and localization, is distributed uniformly and exclusively along the plasma membrane (Brady and O'Halloran, unpublished). The epsin NH_2-terminal domain construct used in this study includes the ENTH domain and a potential clathrin binding motif (Brady and O'Halloran, unpublished). It is possible that expression of the epsin NH_2-terminal construct is sufficient to recruit clathrin and necessary accessory proteins to designated sites of the plasma membrane which allow for partial Hip1r localization and phosphorylation. Alternatively, the expression and localization of the epsin ENTH:GFP chimera to the plasma membrane could alter the

composition of specific regions of membrane in such a way that would allow for a more stable association of Hip1r with the cell periphery. In a variety of organisms, epsin plays a key role during endocytosis and is thought to function as a clathrin adaptor by simultaneously binding the membrane via the ENTH domain and binding clathrin and clathrin accessory proteins via motifs in the COOH-terminal domain (Itoh, et al., 2001; Wendland, 2002; Ford et al., 2002). Recently, the epsin ENTH domain alone, independent of the COOH-terminal domain, was shown to supply clathrin-independent function and interact with the actin-Cdc42 pathway in yeast (Aguilar et al., 2006). Conceivably, in *Dictyostelium* cells, regulation of the actin cytoskeleton by epsin, particularly the ENTH domain, could indirectly influence Hip1r function by altering its interaction with the Hip1r THATCH domain.

Our results demonstrate the importance of the interaction between Hip1r and epsin, a potential functional relationship that cannot be fulfilled by other genes in *Dictyostelium* Hip1r or in epsin null mutants. Like other Hip1r orthologs, *Dictyostelium* Hip1r associates with clathrin punctae on the plasma membrane. The absence of clathrin deficiencies in *Dictyostelium* Hip1r null cells suggests Hip1r could share functional redundancies with other clathrin adaptors in *Dictyostelium* cells. Nonetheless, the dependence on epsin for Hip1r phosphorylation and localization cannot be covered by other adaptors in amoeba. Our identification of a previously unknown potential functional relationship between Hip1r and epsin suggests that a contribution of epsin to Hip1r function be examined in other organisms.

Chapter 3

Contribution of Individual *Dictyostelium* Hip1r Domains to Protein Localization and Function

3.1 INTRODUCTION

In all living organisms, endocytosis is a necessary and frequent event occurring at the membrane of individual cells. Cells receive nutrients, extracellular signals and survey their environment by the transport of material across the plasma membrane. The process of endocytosis, and for that matter, most membrane trafficking processes, is regulated by both the cargo taken in or transported, and the cellular proteins involved in the execution of the event. Clathrin mediated endocytosis is defined as the internalization of material into the cell via clathrin-coated vesicles (Kirchhausen, 2000; Brodsky et al., 2001). The formation of clathrin-coated vesicles is a multi-step process that is dependent upon not only clathrin, but a myriad of cellular proteins that associate with the plasma membrane, clathrin and in some instances, F-actin (Lafer, 2002; Owen et al., 2004; Qualmann and Kessels, 2002; Smythe and Ayscough 2006).

Proteins involved in regulating the formation of a clathrin-coated vesicle are classified as clathrin accessory proteins, meaning they promote formation of a clathrin coat around the membrane that will invaginate and form a vesicle in addition to promoting release of the clathrin coated structure into the cytoplasm (Owen et al., 2004; Maldonado-Báez and Wendland, 2006). The contribution of each protein to clathrin

function and trafficking events are the focus of current research. Understanding how individual factors function in this complex process is constantly being refined. To date, the interactions of clathrin accessory proteins with each other remain unclear. As deposition of clathrin triskelions occurs, the membrane is induced to curve inward, to form a pit or invagination. This process is thought to be mediated by epsin, a clathrin adaptor found in a variety of organisms (Wendland et al., 1999; Ford et al., 2002). A clathrin adaptor is a protein that links clathrin triskelions or assembled clathrin to the cytoplasmic face of the plasma membrane. Many clathrin accessory proteins, including adaptors, also act during the later stages of vesicle formation, such as vesicle scission from the plasma membrane and release into the cytoplasm. The actual act of vesicle scission and release is thought to involve not only clathrin associated proteins but also F-actin. To date, only one family of proteins, the Sla2/Hip1 family, has been shown to interact with both clathrin and actin and thus postulated to act as adaptors during this late stage of endocytosis.

I previously characterized a new member of the Sla2/Hip1 family, *Dictyostelium* Hip1r and identified a functional interaction between Hip1r and another clathrin adaptor, epsin. Epsins have been demonstrated to function at the plasma membrane during clathrin-mediated endocytosis (Chen et al., 1998; Wendland et al., 1999; Wendland, 2002). Like other epsins, the single *Dictyostelium* epsin ortholog contains an ENTH (Epsin NH_2-Terminal Homology) domain that is thought to bind the plasma membrane in regions rich in phosphotidyl inositols. In addition, *Dictyostelium* epsin contains binding

sites for clathrin and other clathrin accessory proteins (Brady and O'Halloran, unpublished).

Our earlier results demonstrated that Hip1r requires epsin for localization and phosphorylation in the social soil amoeba (Repass et al., submitted). Moreover, our previous study identified a requirement for *Dictyostelium* Hip1r during development, a new function for Sla2/Hip1 family members. Specifically, Hip1r is necessary for spore morphology and viability during the development of multicellular *Dictyostelium* fruiting bodies (Repass et al., submitted).

To characterize *Dictyostelium* Hip1r more fully, I explored the relationship between Hip1r and epsin using a variety of methods. First, to examine the contribution of the individual Hip1r domains to *in vivo* localization and function of the protein, I generated three expression plasmids that fuse specific Hip1r domains to either the Green Fluorescent Protein (GFP) or the FLAG epitope. We identified the central coiled-coil domain of Hip1r as an important determinant for its phosphorylation and localization into plasma membrane punctae. In addition, I generated a Hip1r/epsin double mutant. We found that the deletion of both Hip1r and epsin results in phenotypes similar to single Hip1r or epsin null mutants in growing and developing *Dictyostelium* cells.

3.2 RESULTS

3.2.1 The Hip1r ANTH and Coiled-coil Domains are Necessary for the Association of *Dictyostelium* Hip1r with Plasma Membrane Punctae

Previous immunostaining experiments with anti-Hip1r serum demonstrated that endogenous full length Hip1r appears as bright, discrete punctae at the plasma membrane, often colocalizing with epsin (Repass et al., submitted). To identify the domains

responsible for this localization and for Hip1r function, I generated expression vectors for a deletion series of the Hip1r protein that fused specific domains with Green Fluorescent Protein (GFP) or the FLAG epitope (Fig. 3.1). I then expressed these constructs in *Dictyostelium* wild type, Hip1r and epsin null cells.

To assess the contribution of the Hip1r ANTH domain to Hip1r function, the first 354 amino acids of NH_2-terminus of Hip1r, including the ANTH domain, were fused to GFP to generate the construct GFP:Hip1r $ANTH_{(1\text{-}354)}$. When expressed in wild type, Hip1r null and epsin null cells, the fusion protein did not appear as punctae within the cell when observed with fluorescence microscopy (Fig. 3.2A-C). Rather, the GFP:Hip1r-$ANTH_{(1\text{-}354)}$ protein appeared completely diffuse and not associated with any membrane or cytoplasmic structure.

Adjacent to the NH_2-terminal ANTH domain, the central portion of the Hip1r protein consists of regions predicted to form coiled-coil structures (Wesp, et al., 1997; Engqvist-Goldstein, 1999; Mishra et al., 2001; Repass et al., submitted). To examine the localization of a fragment that included the ANTH and central coiled domains, I expressed an NH_2-terminal fragment that included these domains fused to GFP, GFP:Hip1r-$ANTH+CC_{(1\text{-}753)}$, and examined expressing cells using fluorescence microscopy. When expressed in wild type and Hip1r null cells, the ANTH-central domain fusion protein manifested as bright punctae localized at the plasma membrane, similar to the endogenous Hip1r localization observed in wild type cells (Fig. 3.2D-E) (Repass et al., submitted).

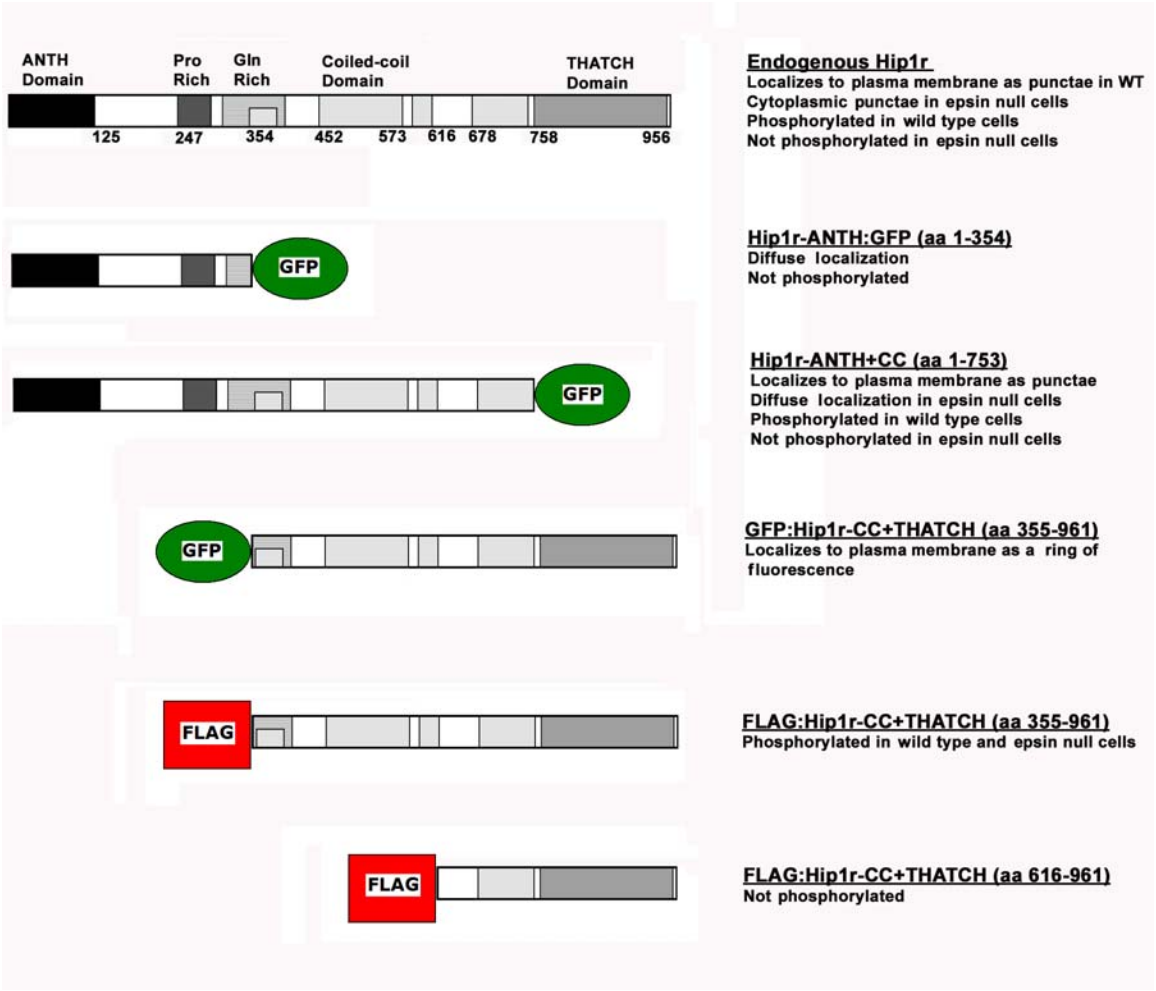

Figure 3.1 Domain Analysis of Hip1r. A series of Hip1r deletion constructs were generated whereby portions of the protein were expressed as either GFP or FLAG-tagged fusion proteins. Endogenous Hip1r has an NH_2-terminal ANTH domain (ANTH, black shading) followed by the central part of the protein containing Proline Rich (Pro Rich, dark gray shading), Glutamine Rich (Gln Rich, striped shading) and multiple coiled-coil motifs (Coiled-coil, light gray shading). The COOH-terminus of the Hip1r protein contains an actin-binding motif, or THATCH domain (THATCH, gray shading). Individual residues that mark the beginning or end of a domain are denoted below the endogenous Hip1r schematic. Regions of overlapping domains are indicated by overlap of shaded boxes. The Hip1r-ANTH:GFP construct consists of the first 354 amino acids from the NH_2-terminus, including the ANTH domain. The Hip1r-ANTH+CC: GFP contains the ANTH region plus all of the coiled-coil domains of Hip1r. The GFP: Hip1r-CC+THATCH or FLAG: Hip1r-CC+THATCH constructs have the NH_2-terminus of Hip1r deleted. The FLAG: 1/3CC+THATCH construct contains the last predicted coiled-coil domain plus the THATCH domain. Localization and/or phosphorylation state of each of the expression vectors are listed on the right.

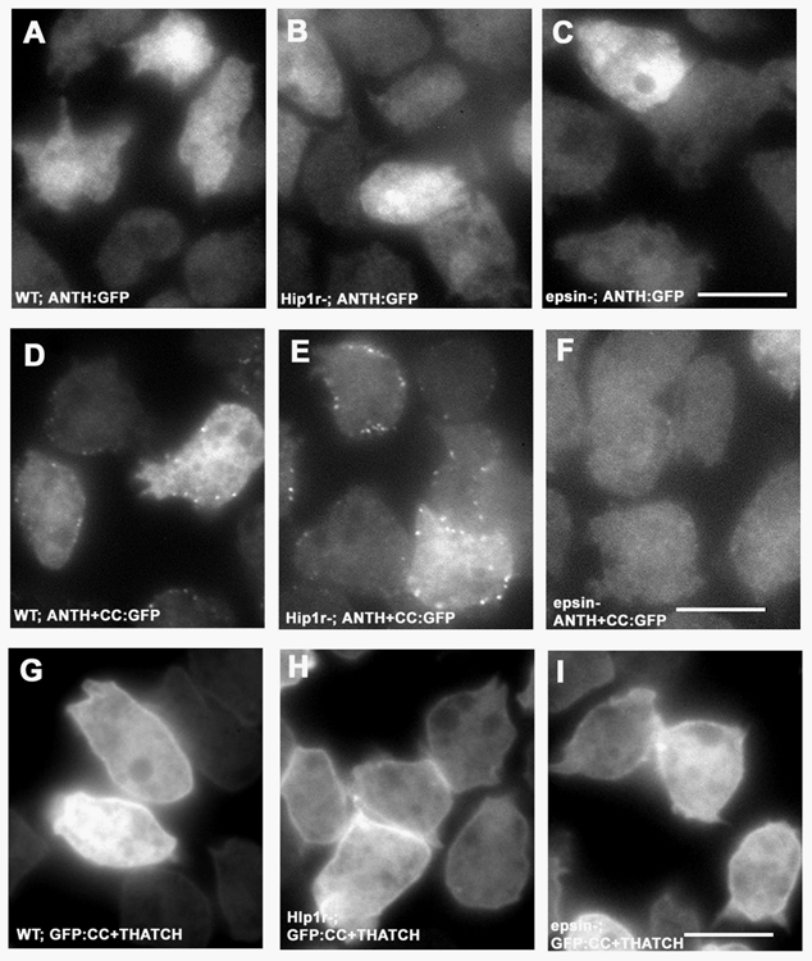

Figure 3.2 The localization of individual Hip1r domains. Constructs of deleted Hip1r domains were generated, fused to GFP and expressed in wild type, Hip1r and epsin null cells. The cells were fixed and viewed with fluorescence microscopy. (A-C) The NH_2-terminal ANTH domain of Hip1r (ANTH:GFP) appears cytosolic and is not restricted to the plasma membrane in wild type (WT, A), Hip1r null (Hip1r-, B) or epsin null (epsin-, C) cells. (D-F) The ANTH plus coiled-coil domain of Hip1r (ANTH+CC:GFP) localizes as punctate fluorescence at the plasma membrane in wild type (D) and Hip1r null (E) cells but appears cytosolic and not associated with the membrane in epsin null cells (E). (G-I) The coiled-coil plus THATCH domain of Hip1r (GFP:CC+THATCH) localizes to the plasma membrane as a continuous ring of fluorescence at the cell periphery in wild type (G) Hip1r null (H) and epsin null (I) cells. Scale bars, 10 μm.

Interestingly, when the GFP:Hip1r-ANTH+CC$_{(1-753)}$ protein was expressed in epsin null cells, the fusion protein did not localize as bright punctae on the plasma membrane as seen in wild type and Hip1r null cells. Instead, the GFP:Hip1r ANTH-CC$_{(1-753)}$ protein appeared diffuse and distributed in the cytoplasm (Fig. 3.2F). The distribution of this fragment in the epsin null cells suggests that both epsin and the ANTH plus coiled-coil domain of Hip1r are required for the association of Hip1r into plasma membrane punctae.

To date, attempts at fusing full length Hip1r to GFP or other tags have been unsuccessful due to difficulties in cloning the entire coding region. For comparison of individual Hip1r domain localization and the full length protein, I rely on my previous immunostaining experiments with affinity purified anti-Hip1r antibodies that demonstrated endogenous Hip1r localizes to plasma membrane punctae as well as cytoplasmic punctae in wild type cells.

3.2.2 The Coiled Domain plus THATCH Domain of *Dictyostelium* Hip1r is Uniformly on the Plasma Membrane.

To assay the contribution of the central coiled region and the THATCH domain in Hip1r localization, I expressed a COOH-terminal fragment that included the coiled domain and putative actin binding, or THATCH, domain as a GFP fusion protein, GFP:Hip1r-CC+THATCH$_{(355-961)}$, in wild type, Hip1r null and epsin null cells. In all cell lines observed, the GFP:Hip1r-CC+THATCH$_{(355-961)}$ protein was restricted to the plasma membrane in a ring of fluorescence at the cell periphery (Fig. 3.2G-I). This staining

pattern contrasted with the endogenous Hip1r pattern in that the distribution was not in punctae. Instead, the fusion protein appeared as a halo of uninterrupted fluorescence, localized at the plasma membrane of wild type, Hip1r null and epsin null cells.

To determine if the GFP: Hip1r-CC+THATCH$_{(754-961)}$ protein was associated with cortical F-actin, I stained cells with fluorescently-labeled phalloidin (Fig. 3.3A-C). Inspection by fluorescence microscopy, revealed that the Hip1r GFP: Hip1r-CC+THATCH $_{(355-961)}$ fusion protein did not colocalize with F-actin at phalloidin-stained structures. To determine whether the GFP: Hip1r-CC+THATCH$_{(355-961)}$ protein localized to the plasma membrane, I incubated cells with the plasma-membrane binding steryl dye FM4-64. All lines exhibited a complete colocalization of the dye and GFP fluorescence around the cell periphery (Fig. 3.3D-E). The distribution of the GFP: Hip1r-CC+THATCH$_{(355-961)}$ protein suggests that the ANTH domain is not required for plasma membrane association. In addition to examining the subcellular localization of Hip1r domains by fluorescence microscopy, I also investigated whether the expressed constructs were able to be phosphorylated in wild type, Hip1r null and epsin null cells.

3.2.3 Epsin and the Coiled-coil Domain of Hip1r are Required for Phosphorylation of Endogenous Hip1r

Endogenous Hip1r is phosphorylated in wild type but not epsin null cells (Repass et al., submitted) (Fig. 3.4A). However, phosphorylation of Hip1r is restored when only the NH_2-terminal epsin ENTH domain is expressed in epsin null cells (Repass, et al., submitted).

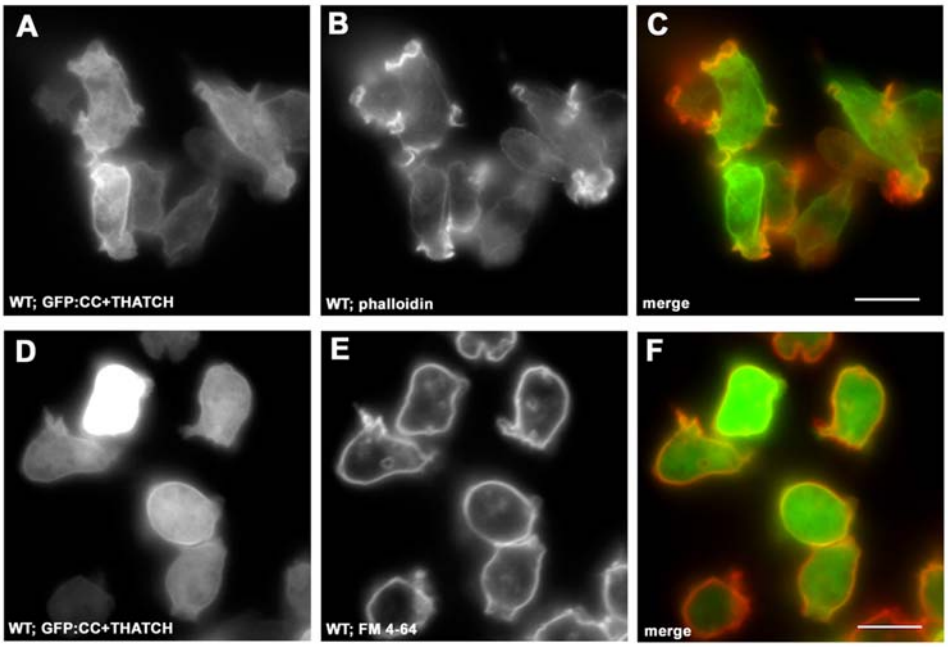

Figure 3.3 The coiled-coil plus THATCH domain of Hip1r localizes to the plasma membrane. (A-C) Wild type cells expressing the NH_2-terminal deletion Hip1r fusion protein (GFP:CC+THATCH $_{(355\text{-}961)}$) were fixed, stained with Texas-Red conjugated phalloidin and viewed with fluorescence microscopy. Wild type cells expressing the GFP:CC+THATCH$_{(355\text{-}961)}$ chimera show localization of the fusion protein to the plasma membrane (A) and contain regions rich in F-actin at the cell cortex (B). However, the GFP:CC+THATCH$_{(355\text{-}961)}$ protein does not colocalize with the cortical F-actin (E). (D-F) Live cells expressing the CC+THATCH$_{(355\text{-}961)}$ fusion protein were incubated with the membrane-staining dye, FM4-64 and viewed with fluorescence microscopy. The CC+THATCH$_{(355\text{-}961)}$ protein is restricted to the plasma membrane (D) and shows overlap with the FM4-64 dye (E, F). Scale bar, 10 μm.

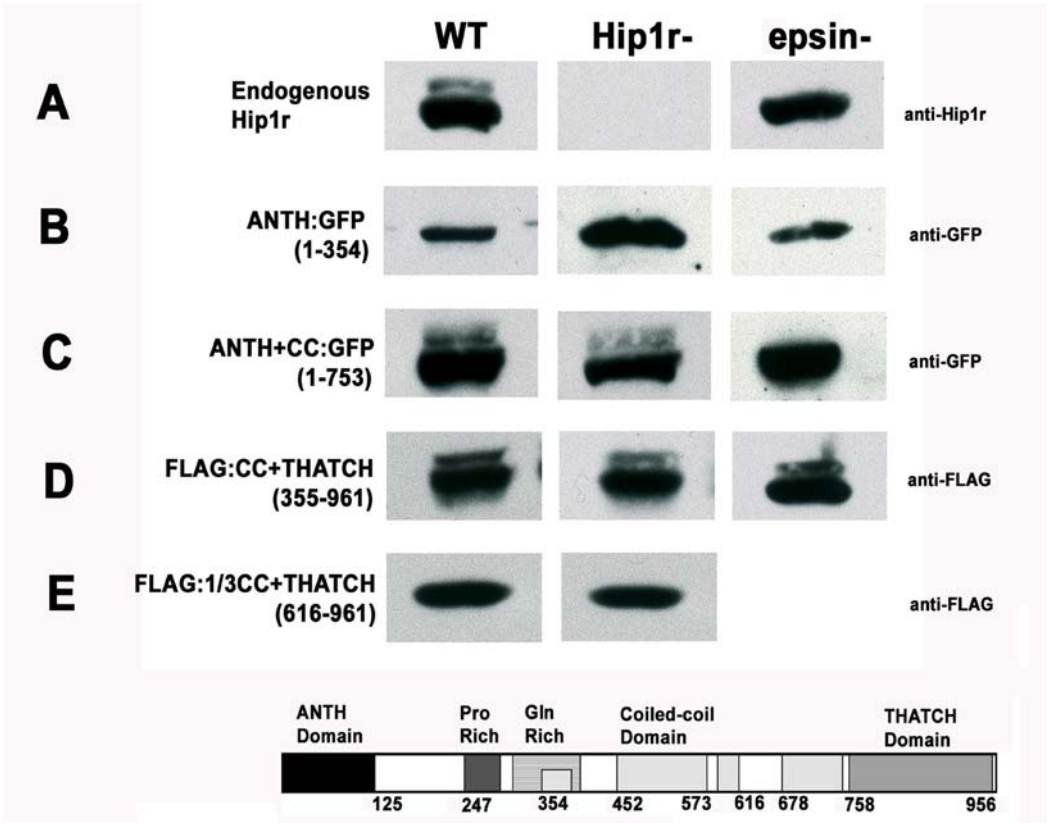

Figure 3.4 Epsin and the coiled-coil domain of Hip1r are required for phosphorylation of the endogenous protein. Lysates of cell expressing Hip1r deletion constructs were subjected to SDS-PAGE and immunoblotted with anti-Hip1r, anti-GFP or anti-FLAG serum. (A) Endogenous Hip1r is phosphorylated in wild type cells (WT), absent in Hip1r null cells (Hip1r-) and not phosphorylated in epsin null cells (epsin-). (B) The Hip1r ANTH domain fused to GFP (ANTH:GFP$_{(1-354)}$) is not phosphorylated in wild type, Hip1r null and epsin null cells as determined by blotting with anti-GFP serum. (C) The Hip1r ANTH domain plus coiled-coil region fused to GFP (ANTH+CC:GFP $_{(1-753)}$) is phosphorylated in wild type and Hip1r null cells, but not in cells lacking epsin as determined by blotting with anti-GFP serum. (D) A FLAG fusion protein with the ANTH domain deleted (FLAG:CC+THATCH $_{(355-961)}$) is phosphorylated in wild type, Hip1r null and epsin null cells, as determined by blotting with anti-FLAG monoclonal antibodies. (E) Loss of the 261 amino acids from the NH_2-terminal portion of the Hip1r coiled-coil domain (FLAG:1/3CC+THATCH $_{(616-961)}$) results in the lack of Hip1r phosphorylation in wild type and Hip1r null cells when the deletion is expressed as a FLAG-tagged fusion protein.

Lysates of cells expressing the GFP:Hip1r-ANTH$_{(1-354)}$ or GFP:Hip1r-ANTH+CC$_{(1-753)}$ fusion proteins were harvested and then analyzed by immunoblotting with anti-GFP antibodies. Analysis of the immunoblots revealed that the GFP:Hip1r-ANTH$_{(1-354)}$ protein migrated as a single band in all cell lines, suggesting the ANTH domain was not phosphorylated (Fig. 3.4B). On the other hand, lysates of wild type and Hip1r null cells expressing the GFP:Hip1r-ANTH+CC$_{(1-753)}$ fusion protein revealed two bands, suggesting that the fusion protein was phosphorylated (Fig. 3.4C). In contrast, only a single band was observed for the GFP:Hip1r-ANTH+CC$_{(1-753)}$ fusion protein in epsin null cells (Fig. 3.4C).

I also analyzed the phosphorylation state of GFP:Hip1r-CC+THATCH $_{(355-961)}$ fusion proteins that were expressed in wild type, Hip1r null and epsin null cells. Because the CC+THATCH domain fused to GFP migrates at a similar size predicted for endogenous Hip1r, we generated a FLAG-tagged construct (FLAG:Hip1r-CC+THATCH$_{(355-961)}$). Lysates of wild type, Hip1r null and epsin null cells expressing the FLAG:Hip1r-CC+THATCH $_{(355-961)}$ proteins were analyzed by immunoblotting with anti-FLAG antibodies (Fig. 3.4D-F). As with the ANTH plus coiled domain, the FLAG:Hip1r-CC+THATCH $_{(355-961)}$ fusion protein was phosphorylated in both wild type and Hip1r null cells. Surprisingly, in contrast with endogenous Hip1r in epsin null cells, the FLAG:Hip1r-CC+THATCH $_{(355-961)}$ fusion protein was also phosphorylated in cells lacking epsin expression.

These results suggested that the coiled domain region of Hip1r was important for the phosphorylation of the protein. To explore this hypothesis in more detail, I designed a FLAG-tagged deletion construct that would express the COOH-terminal portion of the

coiled-coil plus THATCH domain of Hip1r (FLAG:Hip1r-1/3CC+THATCH $_{(616-961)}$). SDS-PAGE analysis of lysates from wild type and Hip1r null cells expressing the FLAG:Hip1r-1/3CC+THATCH$_{(616-961)}$ construct were immunoblotted with anti-FLAG antibodies (Fig. 3.4G). A single band at the predicted size for the FLAG:Hip1r-1/3CC+THATCH$_{(616-961)}$ fusion protein was observed, suggesting that the entire coiled domain is required for Hip1r phosphorylation.

3.2.4 Full Length Hip1r is Essential for Proper Spore Morphology in *Dictyostelium*

When *Dictyostelium* cells are starved for nutrients, they will initiate a developmental program whereby cells will aggregate and form a multicellular fruiting body. The fruiting body consists of a thin stalk supporting a sorus. Within the sorus are spores--dormant amoebae that are encased in a thick outer coat designed to withstand harsh environmental conditions. Wild type cells produce ovoid-shaped spores with robust spore coats. Cells lacking functional Hip1r produce round, rather than ovoid, spores with reduced viability (Repass et al., submitted).

Using the expression vectors for the Hip1r proteins, I investigated whether any of the fusion proteins could rescue the abnormal spores produced by cells lacking Hip1r expression. Hip1r null cells expressing all of the fusion proteins formed fruiting bodies, similar to those formed by either wild type or Hip1r null cells (data not shown). Examination of the spores housed within the sorus of the fruiting bodies revealed that all strains formed round spores (Fig. 3.5A-E). This finding implies that full length Hip1r is required for the production of ovoid-shaped spores in *Dictyostelium*.

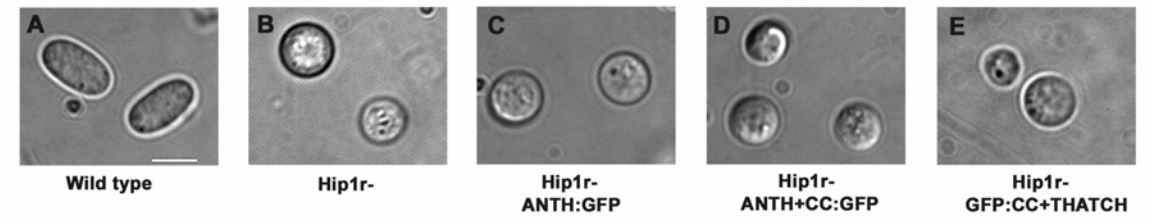

Figure 3.5 Full length Hip1r is required for proper spore morphology. Spores were harvested from fruiting bodies derived from wild type and Hip1r null cells expressing the Hip1r domain fusion proteins and viewed with DIC microscopy. (A) Wild type spores display an elliptical morphology. (B-E) Hip1r null (Hip1r-) or Hip1r null cells expressing one of the three Hip1r deletion proteins exhibit the round spore phenotype. Scale bar, 5 μm.

3.2.5 Loss of Hip1r and Epsin Expression Does Not Affect Viability or Clathrin Localization in Growing *Dictyostelium* Cells

I demonstrated a functional interaction of Hip1r and epsin in both growing and developing cells in *Dictyostelium* (Repass et al., submitted). Hip1r requires epsin for phosphorylation and localization to plasma membrane punctae. The expression of the epsin NH_2-terminal ENTH domain is sufficient to restore the localization of Hip1r to plasma membrane punctae as well as the phosphorylation of the protein in epsin null cells (Repass et al., submitted). In developing cells, both Hip1r null and epsin null cells produce spores with similar abnormal morphology, although the phenotype is less severe in epsin null cells (Repass et al., submitted; Brady and O'Halloran, unpublished). While expression of the epsin ENTH domain is sufficient to restore wild type spore morphology in epsin null cells, the epsin ENTH domain does not rescue the round spore phenotype of the Hip1r mutant cells (Brady and O'Halloran, unpublished; Repass and O'Halloran, unpublished observation). Based on these findings, I postulated that epsin acts upstream of Hip1r and thereby regulates Hip1r localization and/or function. If this is true, Hip1r should require epsin to function properly whereas Hip1r would not be expected to influence epsin function in *Dictyostelium*.

In order to test this hypothesis, I constructed a double mutant that harbored a deletion of both the Hip1r and epsin genes. Deletion of the *hipA* gene was accomplished by homologous recombination and subsequent replacement of *hipA* with an integrating selectable marker. Once a single Hip1r null strain was obtained, these cells were cultured

and assayed for the absence of Hip1r by immunoblotting with anti-Hip1r serum (Fig. 3.6 A). The Hip1r null cells were subjected to a second round of homologous recombination using a second, distinct selectable marker to induce the deletion of the *epnA* gene. The resulting cells were cultured and assayed for the absence of both Hip1r and epsin by immunoblotting with anti-Hip1r serum and anti-epsin serum, respectively (Fig. 3.6B).

Single Hip1r and epsin nulls cells are viable and the double mutant, Hip1r/epsin null cells were also viable. In *Dictyostelium*, the inability to complete cytokinesis in suspension is one of the hallmarks of clathrin as well as other membrane trafficking deficiencies (O'Halloran and Anderson, 1992; Niswonger and O'Halloran, 1997; Kwak et al., 1999**).** When the Hip1r/epsin mutant cells were grown in suspension, the amoebae were able to efficiently complete cytokinesis (data not shown).

Since both Hip1r and epsin are known clathrin adaptors, I examined the Hip1r/epsin mutant cells for mislocalization of endogenous clathrin. The parental wild type strain and Hip1r/epsin mutant cells were fixed and immunostained with affinity-purified anti-clathrin antibodies. When examined with fluorescence microscopy, both cell lines displayed a similar quantity and intensity of clathrin punctae at the plasma membrane as well as the characteristic juxtanuclear staining observed for clathrin (Fig. 3.7A-B). Both Hip1r and epsin orthologs have been shown to associate with and/or potentially regulate F-actin (Wesp et al., 1997; Yang et al., 1999; Engqvist-Goldstein et al., 1999; Engqvist-Goldstein et al., 2004; Aguilar et al., 2006). I examined the

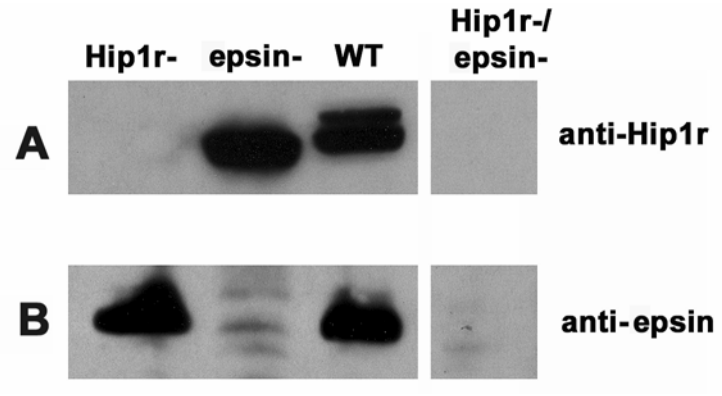

Figure 3.6 Generation of a Hip1r/epsin double mutant strain. (A-B) The absence of Hip1r and epsin expression was confirmed by immunoblotting whole cell lysates of Hip1r mutant cells (Hip1r-), epsin mutant cells (epsin-), wild type cells (WT) and Hip1r/epsin mutant cells (Hip1r-/epsin-) with anti-Hip1r (A) and anti-epsin (B) polyclonal serum.

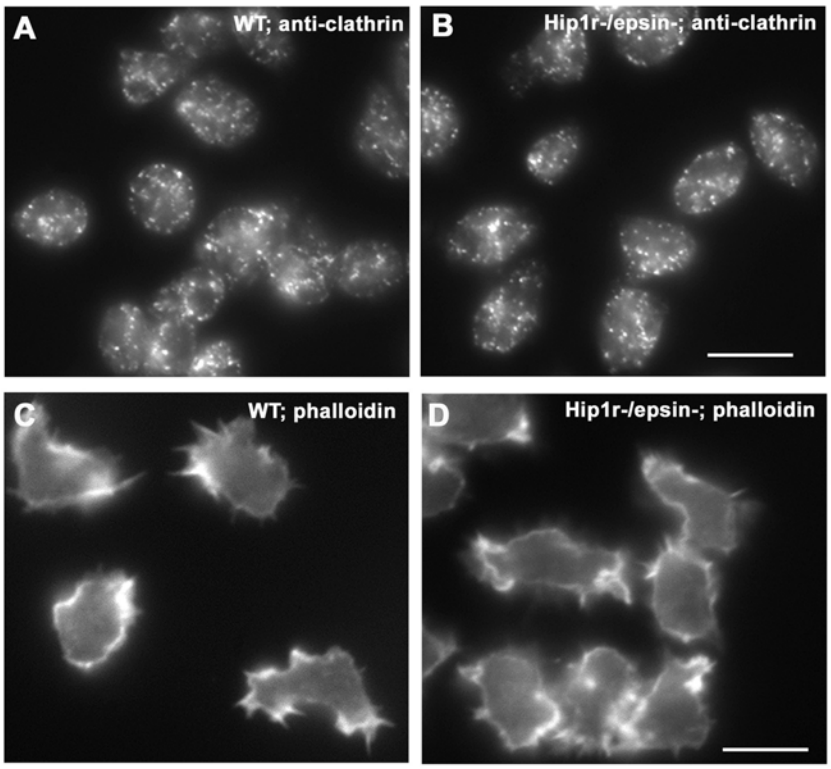

Figure 3.7 The Hip1r/epsin double mutant displays normal clathrin and cortical F-actin. (A-B) Wild type and Hip1r/espin mutant cells were fixed and stained with anti-clathrin antibodies. Both wild type (WT) and mutant (Hip1r-/epsin-) show punctae at the plasma membrane and juxtanuclear staining. (C-D) To visualize F-actin, wild type and Hip1r/espin mutant cells were stained with Texas Red-conjugated phalloidin. Both wild type (WT) and Hip1r/epsin mutant cells (Hip1r-/epsin-) show staining of F-actin at the cell cortex. Scale bars, 10μm

Dictyostelium Hip1r/epsin mutant cells for potential defects in F-actin organization by staining wild type and mutant cells with fluorescently-labeled phalloidin. When viewed with fluorescence microscopy, the Hip1r/epsin null cells displayed a substantial band of F-actin at the cortex of the cell that was indistinguishable from wild type, suggesting that the absence of Hip1r and epsin does not adversely affect the overall organization of cortical actin (Fig. 3.7C-D).

3.2.6 Hip1r/ Epsin Mutant Cells Yield Spores Identical to Hip1r Mutant Spores

When *Dictyostelium* cells are faced with a diminution of nutrients from their surrounding medium, they will commence a developmental program that results in the production of dormant amoeba encased in a tough, trilaminar spore coat that is comprised of glycoproteins and organized cellulose. These spores are the end product of a series of events that begins with the aggregation of approximately 1×10^5 individual starving amoebae. The aggregating cells will converge and eventually form a fruiting body--a multicellular structure that consists of a slender stalk supporting a sorus which houses the spores. The dormant amoebae will shed their spore coats to produce viable cells when environmental conditions are favorable.

In order to test our hypothesis that epsin acts upstream of Hip1r, I examined the developmental structures of the Hip1r/epsin double mutant. I postulated that the deletion of both proteins would not have a synergistic effect but rather, resulting phenotypes would mirror that of the single mutants. The absence of either Hip1r or epsin does not affect fruiting body formation or structure (Repass et al., submitted; Brady and

O'Halloran, unpublished). Wild type and Hip1r/epsin mutant cells were inoculated onto non-nutrient agar plates. The Hip1r/epsin mutant cells were able to aggregate normally and formed fruiting bodies comparable to structures formed by wild type cells, similar to results observed for the single Hip1r and epsin null mutants (Fig. 3.8A-D).

Both Hip1r and epsin null cells give rise to spores with abnormal morphology, although the phenotype is less severe in epsin null cells. To test if the absence of both Hip1r and epsin influences spore morphology, I compared spores derived from Hip1r/epsin mutant fruiting bodies with those derived from either the single mutants or the parental wild type strain. When viewed under DIC microscopy, the spores from the Hip1r/epsin mutant cells were identical in morphology to spores made by Hip1r null cells. That is, the Hip1r/epsin mutant spores were completely round and able to synthesize cellulose as measured by staining the mutant spores with calcofluor, a cellulose-binding reagent (Fig. 3.8E-L).

To assay their viability, spores from the Hip1r/epsin mutants, the parental wild type strain, single Hip1r null and epsin null cells were collected. After washing with either detergent or treating with heat, the spores were incubated with bacteria on agar plates to assess their viability. When washed the non-ionic detergent NP-40 or heated briefly to 45°C, both Hip1r null spores and Hip1r/epsin mutant spores displayed a survival rate that was approximately half that of wild type and epsin null spores (data not shown). Thus Hip1r/epsin mutant spores did not display more pronounced defect of spore coat strength relative to Hip1r null spores suggesting that loss of both Hip1r and epsin protein expression does not give way to a more defective spore coat.

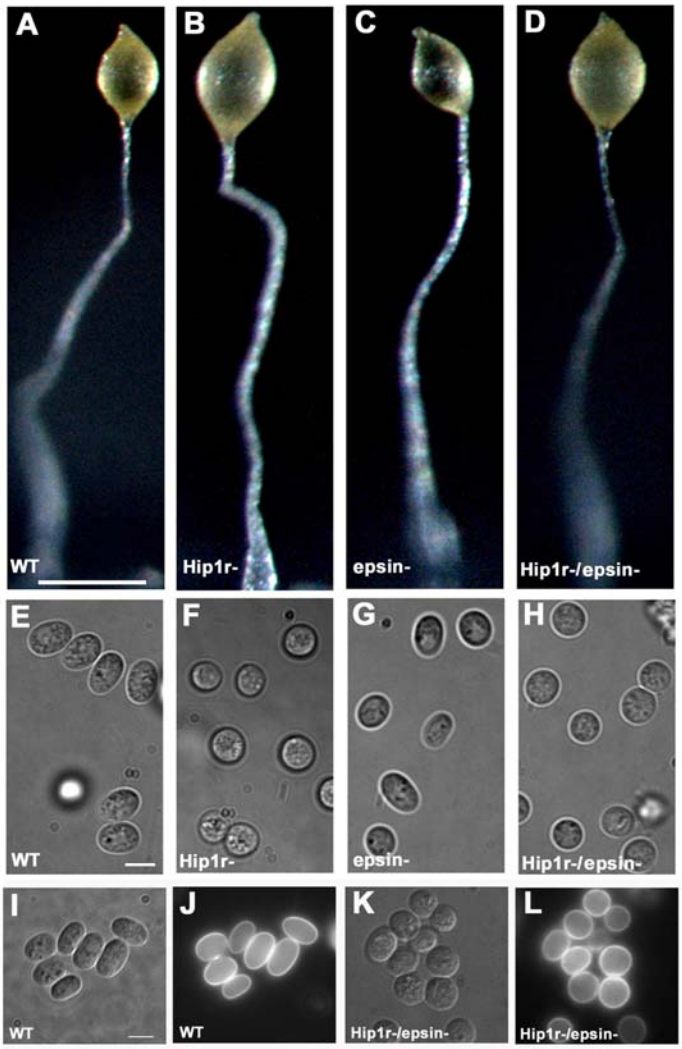

Figure 3.8 Hip1r/epsin mutant cells display similar developmental phenotypes as those observed for Hip1r single mutant cells. Wild type, Hip1r null cells, epsin null cells and Hip1r/epsin mutant cells were harvested and inoculated onto non-nutrient agar plates to induce development of fruiting bodies and spores. (A-D) Wild type (WT, A), Hip1r null (Hip1r-, B), epsin null (epsin-, C) and Hip1r/epsin mutant cells (Hip1r-/epsin-, D) all form fruiting bodies of similar size and structure. Scale bar, 0.25 mm. (E-F) Spores were harvested from wild type, Hip1r null, epsin null and Hip1r/epsin mutant fruiting bodies and examined with DIC microscopy. Wild type spores (E) are ovoid whereas Hip1r null (F) and Hip1r/epsin mutant spores display the same round morphology. Epsin null spores (G) are a mixture of round and ovoid spores. Scale bar, 5μm. (I-L) Wild type and Hip1r/epsin mutant spores were harvested, stained with calcoflour, a cellulose-staining dye, and viewed under differential interference contrast (DIC) or fluorescence microscopy. Both wild type and the double mutant spores are cellulose-positive. Scale bar, 5μm.

3.3 DISCUSSION

The findings presented in this study identify the Hip1r domains that are necessary for the phosphorylation of the protein in addition to its association with the plasma membrane. I further characterize the interaction between two *Dictyostelium* clathrin adaptors, Hip1r and epsin. Epsin and the ANTH plus coiled-coil domains of Hip1r are necessary for the localization of the Hip1r protein in plasma membrane punctae. Moreover, the NH_2-terminal portion of the Hip1r coiled-coil region is necessary for phosphorylation of the Hip1r protein. However, full length Hip1r is required for the formation of spores with wild type morphology. Finally, while the loss of both Hip1r and epsin expression does not hinder growth or development of the organism, the absence of both proteins yields developmental phenotypes analogous to cells lacking only Hip1r or epsin expression.

3.3.1 Epsin and the Coiled-coil Domain of Hip1r are Required for Localization of Hip1r into Membrane Punctae

Previously I identified a role for epsin, particularly the NH_2-terminal ENTH domain of epsin, in Hip1r localization and phosphorylation in *Dictyostelium* (Repass et al., submitted). I set out to determine which portions of the Hip1r protein were necessary for localization and function as well as to identify which Hip1r domains may be essential for the interaction of the protein with epsin.

The NH_2-terminal ANTH domain has been shown to be essential for endocytosis in yeast whereas in mammalian cells, the Hip1r ANTH domain alone does not localize to any particular cellular structure (Wesp et al., 1997; Engqvist-Goldstein et al., 1999). I generated three deletion constructs of Hip1r fused to GFP to monitor localization of the domains by fluorescence microscopy. I am unable to compare the localization of individual Hip1r domain constructs with full length Hip1r tagged with GFP as attempts at generating such a construct have been unsuccessful. For comparison of localization of individual domains and the full length protein, I rely on my previous finding that demonstrated endogenous Hip1r localizes to plasma membrane and cytoplasmic punctae in wild type cells (Repass et al., submitted). When the Hip1r ANTH domain was expressed as a GFP fusion protein in *Dictyostelium,* it did not appear as membrane punctae but rather diffuse and mostly cytosolic. Moreover, in *Dictyostelium,* expression of only the ANTH domain was insufficient for phosphorylation of the Hip1r fragment. On the other hand, the ANTH domain plus full coiled-coil region of Hip1r localized to the plasma membrane in the form of bright punctae in both wild type and Hip1r null cells. In fact, this localization as plasma membrane punctae was identical to that observed when endogenous Hip1r is immunostained with anti-Hip1r serum in wild type cells. Conversely, expression of the ANTH plus coiled-coil region of Hip1r in *Dictyostelium* epsin null cells did not result in plasma membrane staining but appeared diffuse and cytosolic. Taken together, these results suggest that while the ANTH domain of Hip1r is predicted to direct the protein to regions of the membrane rich in phosphotidylinositol-

4,5-bisphosphate (PIP_2), it is the association of Hip1r with epsin that allows for Hip1r inclusion into plasma membrane punctae.

3.3.2 The NH_2-terminal Portion of the Coiled-coil Domain of Hip1r is Necessary for Phosphorylation

While the GFP:Hip1r-ANTH+$CC_{(1-753)}$ fusion protein was phosphorylated in wild type and Hip1r null cells, this fragment was not phosphorylated in epsin null cells. In contrast, the FLAG: Hip1r-CC+THATCH $_{(355-961)}$ chimeric protein is phosphorylated in not only wild type and Hip1r null cells, but epsin null cells as well. In addition, rather than localizing as plasma membrane or cytoplasmic punctae, the FLAG: Hip1r-CC+THATCH $_{(355-961)}$ chimeric protein localizes as a ring of fluorescence around the periphery of the cell. The localization of the Hip1r coiled-coil plus THATCH domain was not unanticipated since studies in mammalian cells have yielded similar results (Engqvist-Goldstein et al., 1999). On the other hand, phosphorylation of the coiled-coil plus THATCH domain was unexpected. Given the localization of the GFP: Hip1r-CC+THATCH $_{(355-961)}$ to the plasma membrane of epsin null cells, this result suggests that Hip1r constructs that localize and associate with the plasma membrane can be phosphorylated even in the absence of epsin.

The uniform association of the FLAG: Hip1r-CC+THATCH $_{(355-961)}$ fusion protein with the plasma membrane suggests the protein is interacting, through the coiled domain and/or F-actin binding domain, with other factors present at the cell periphery which influence the phosphorylation state of Hip1r. However, the ANTH domain of the protein, in conjunction with the Hip1r coiled-coil domain and epsin, increases Hip1r affinity for plasma membrane punctae.

To explore the regions of the Hip1r protein that are necessary for phosphorylation, I constructed an expression vector, FLAG: Hip1r-1/3CC+THATCH **(616-961)**, that lacked the ANTH domain and approximately two-thirds of the coiled domains of Hip1r. I found that the expressed proteins were not phosphorylated in either wild type or Hip1r null cells. Whether the deleted amino acids contain the phosphorylation site for an unidentified kinase or if the entire coiled-coil region could be necessary for appropriate protein folding and subsequent phosphorylation remains to be determined.

3.3.3 Comparison of *Dictyostelium* Domain Analyses with that of Other Sla2/Hip1 Family Members

The *Dictyostelium* Hip1r domain constructs demonstrated a similar localization pattern as those observed for other orthologs, specifically yeast Sla2p and mammalian Hip1r (Yang et al., 1999; Engqvist-Goldstein et al., 1999). Endogenous Sla2p localizes to cortical actin patches, active sites for endocytic event in budding yeast (Yang et al., 1999). In both mammalian and *Dictyostelium* cells, endogenous Hip1r localizes to the plasma membrane as punctate fluorescence with some cytoplasmic staining however, unlike mammalian Hip1r, the *Dictyostelium* ortholog does not exhibit juxtanuclear staining (Engqvist-Goldstein et al., 1999; Repass et al., submitted).

When the NH_2-terminal ANTH domain of the orthologs were expressed as GFP fusion proteins, both mammalian and *Dictyostelium* Hip1r appeared diffuse and cytosolic (Engqvist-Goldstein et al., 1999; this study). In budding yeast, the ANTH domain of Sla2p alone still localizes to cortical actin patches but exhibits some cytoplasmic staining (Yang et al., 1999). A similar staining pattern is observed when a large portion (amino

acids 33-359) of the NH_2-terminal region of Sla2p is missing (Yang et al., 1999). On the other hand, when the ANTH domain is deleted in both *Dictyostelium* and mammalian cells, the remainder of the Hip1r protein--the central coiled region plus THATCH domain--localizes to the cell periphery as a halo of fluorescence (Engqvist-Goldstein et al., 1999; this study). Finally, expression of the ANTH plus coiled-coil region, without the THATCH domain, has been shown to be sufficient for localization into membrane punctae in both yeast and mammalian family members (Yang et al., 1999; Engqvist-Goldstein et al., 1999). As in other systems, expression of the *Dictyostelium* ANTH plus central coiled-coil domains restored the localization of the fusion protein to membrane punctae, a staining pattern observed for endogenous Hip1r in wild type cells.

Although Sla2p/Hip1 family members appear to be phosphorylated in other systems, I am unable to compare the phosphorylation status of the expressed *Dictyostelium* deletion constructs with those used in other organisms. To the best of our knowledge, the present study represents the first attempt to identify the role individual Hip1r domains play in the phosphorylation of the protein.

3.3.4 The Absence of Both Hip1r and Epsin Does Not Have a Synergistic Effect

I have previously demonstrated that the absence of epsin interferes with Hip1r localization and phosphorylation but the absence of Hip1r does not affect epsin localization in *Dictyostelium* (Repass et al., submitted). In developing cells, both Hip1r and epsin null cells produce round spores, although the epsin null phenotype is less severe. While Hip1r null spores are completely round, the epsin null spores range in size from round to elliptical, the morphology observed for wild type spores (Repass et al.,

submitted; Brady and O'Halloran, unpublished). In addition, Hip1r null spore coats have disorganized cellulose. The similar spore morphology and the lack of Hip1r association with plasma membrane punctae in epsin null cells suggests that the round spore phenotype results from the absence of Hip1r at the cell cortex. If this hypothesis were true, deleting both proteins should result in phenotypes analogous to those observed for Hip1r or epsin mutant cells. In order to test this hypothesis I constructed double mutant cell line.

Similar to the single Hip1r null or single epsin null mutant cells, the Hip1r/epsin double mutant cells did not display a mislocalization of endogenous clathrin or exhibit gross abnormalities in the organization of F-actin at the cell cortex. During development, the Hip1r/epsin double mutant cells formed fruiting bodies however, the double mutant cells gave rise to completely round, rather than ovoid, spores. Moreover, the spores harvested from the Hip1r/epsin mutant fruiting bodies displayed a similar survival rate that was similar to single Hip1r mutant spores when tested under adverse conditions.

Analysis of individual Hip1r domain function, together with observations of Hip1r/epsin double mutant phenotypes, leads us to conclude that full length Hip1r is required for proper spore formation and that the Hip1r protein must be associated with the plasma membrane for the production of viable spores with wild type morphology. Furthermore, I propose the defect observed in developing epsin null cells is due to the mislocalization of Hip1r, and not solely due to the loss of epsin.

3.3.5 Hip1r and Epsin in *Dictyostelium* Cells

The present study yields insight into the interaction between clathrin adaptors, Hip1r and epsin, and their role in processes occurring at the plasma membrane that have yet to be described in other systems. The domain analysis of Hip1r allows us to formulate a potential model for the association of Hip1r with the plasma membrane and the interaction of the protein with epsin in *Dictyostelium*. I propose that Hip1r is recruited to the plasma membrane and through the ANTH and coiled-coil domain, Hip1r is "stabilized" or "clustered" in the form of punctae in regions of the plasma membrane rich in phosphotidyl inositols. This clustering of Hip1r requires both epsin and the Hip1r coiled-coil domain. The epsin-dependent phosphorylation requires the NH_2-terminal region of the Hip1r coiled-coil domain. In developing cells, full length Hip1r is required for normal spore shape. In addition, I propose epsin null cells exhibit a defect in spore morphology because Hip1r is mislocalized in these cells. Conceivably, Hip1r could associate with the plasma membrane through its ANTH domain and cortical actin via the THATCH domain while simultaneously interacting with epsin and/or other membrane-associated proteins through the Hip1r coiled-coil region. At this point, the exact method of Hip1r action becomes less clear. Hip1r could be aiding in the late stages of vesicle formation by acting as a scaffold for other accessory proteins of the endocytic complex. Alternatively, through the THATCH domain, Hip1r could assist in the release of the mature vesicle from the membrane by influencing actin dynamics at the site of scission. Studies with mammalian Hip1r have shown that the protein, in conjunction with cortactin, another clathrin accessory protein, acts as a negative regulator of actin

dynamics during vesicle formation by inhibiting filament elongation near the clathrin coat and funneling actin monomers to new filament assembly at the vesicle neck (Le Clainche et al., 2007). Conceivably, the phosphorylation of Hip1r described in the present study could provide the mechanism by which the protein regulates actin dynamics during vesicle maturation and scission. In any instance, the *Dictyostelium* Hip1r protein and its dependence upon epsin for phosphorylation and localization provide insights into the relationship between clathrin associated proteins as well as the mechanism of clathrin-mediated events in eukaryotic cells.

Chapter 4

Conclusions and Future Directions

Members of the Sla2/Hip1 family of adaptor proteins are postulated to act as molecular links between the endocytic machinery and the actin cytoskeleton during clathrin-mediated endocytosis. While analyses of yeast Sla2p and mammalian Hip1 and Hip1r supports this hypothesis, the mechanism of Sla2/Hip1r function during membrane trafficking events and other cellular processes has yet to be elucidated. I have identified a *Dictyostelium discoideum* ortholog of Hip1r. In this study, I have characterized the localization and function of the protein in growing and developing amoeboid cells and described phenotypes that result from loss of expression of the Hip1r protein. I have demonstrated that Hip1r displays a potential functional interaction with another clathrin adaptor, epsin. In addition, I have shown that Hip1r is required for the production of robust spores with wild type morphology during multicellular development of *Dictyostelium*. This investigation raises significant questions regarding the interactions between clathrin associated proteins during membrane events as well as the contribution of such proteins to developmental processes.

4.1 HIP1R DISPLAYS A POTENTIAL FUNCTIONAL INTERACTION WITH EPSIN IN *DICTYOSTELIUM DISCOIDEUM*

4.1.1. Epsin is Required for Hip1r Localization to Plasma Membrane Punctae

I have demonstrated the *Dictyostelium* ortholog of Hip1r interacts with a known clathrin adaptor, epsin. This is a novel association between two clathrin associated proteins that has not been described in other systems. Endogenous Hip1r localizes to the plasma membrane in wild type cells and extensively colocalizes with epsin in membrane punctae. In the absence of epsin, Hip1r is not restricted to plasma membrane punctae nor is Hip1r phosphorylated (Chapter 2, this study). Domain analysis of Hip1r demonstrated that, in addition to the presence of epsin, the ANTH plus central coiled region of Hip1r is necessary to localize Hip1r into plasma membrane punctae (Chapter 3, this study). Conversely, expression of the Hip1r ANTH domain alone was not sufficient to restrict the protein to plasma membrane punctae in wild type or epsin null cells. These results suggest that the central coiled domain of Hip1r mediates the interaction between Hip1r and epsin. Whether this is a direct or an indirect interaction remains to be determined. To begin to test this, I have preliminary results from immunoprecipitation experiments that assessed lysates of *Dictyostelium* cells incubated with anti-Hip1r antibodies (data not shown). Epsin was absent from the fractions bound by the anti-Hip1r antibody when samples were subjected to SDS-PAGE and immunoblotted with anti-epsin antibodies, suggesting the interaction between the two proteins is indirect or of low affinity.

Recent data from studies with *Saccharomyces cerevisiae* have reported a direct interaction between the yeast Hip1r ortholog, Sla2p, and Pan1p, the yeast ortholog of

Eps15 (Toshima, et al., 2007; Wendland, et. al., 1996). Pan1p is an essential protein in yeast as it is required for internalization and F-actin organization during endocytosis (Tang and Cai, 1996; Wendland et al., 1996). Pan1p has been demonstrated to bind directly to the yeast homologs of epsin, Ent1 and Ent2 (Wendland and Emr, 1998; Wendland et al., 1999). It is attractive to speculate that the Hip1r and epsin interaction observed in *Dictyostelium* cells could be mediated through the *Dictyostelium* ortholog of Pan1p. Search of the *Dictyostelium* database reveals the presence of at least three potential Pan1p candidates. A detailed characterization of these potential Pan1p orthologs, along with other potential clathrin accessory proteins, would advance our understanding of the Hip1r and epsin interaction in *Dictyostelium* as well as other organisms. On the other hand, the role of clathrin as a potential mediator of Hip1r and epsin interaction should be examined in more detail as studies in other organisms have shown clathrin binds directly to both Hip1r and epsin orthologs and thus may act as a link between the two adaptors (Engqvist-Goldstein et al., 2001; Henry et al., 2002; Chen et al., 1998; Kay et al., 1999).

4.1.2 Epsin Influences Hip1r Phosphorylation

In Chapter 2 I demonstrated that epsin is required for Hip1r phosphorylation. Phosphorylation events play a key role in the formation of clathrin coats as the function of multiple clathrin accessory proteins are regulated in this manner (Brodsky et al., 2001; Hao et al., 1999; Wilde and Brodsky, 1996). While other members of the Sla2/Hip1 family are phosphorylated (Yang et al., 1999), this is the first investigation describing

conditions necessary for the phosphorylation of Hip1r. Endogenous Hip1r and Hip1r deletion constructs expressing the ANTH domain plus central coiled domain (GFP:Hip1r-ANTH+CC$_{(1-753)}$) are phosphorylated in wild type cells but not in epsin null cells. Both endogenous Hip1r and the fusion protein localize as punctae at the plasma membrane in wild type cells. The dependence upon epsin for Hip1r phosphorylation in *Dictyostelium* cells, taken together with the mislocalization of Hip1r in the absence of epsin, suggests that epsin influences the positioning of endogenous Hip1r such that the unidentified kinase is able to phosphorylate Hip1r at the membrane.

While epsin was required for full length Hip1r phosphorylation and localization, a Hip1r deletion construct lacking the ANTH domain (GFP:Hip1r-CC+THATCH $_{(355-961)}$) localized completely to the plasma membrane and was phosphorylated in all cell lines, including epsin null cells. Conceivably, because the fusion protein associates with the plasma membrane it could be phosphorylated even in the absence of epsin by virtue of its close proximity to the unidentified kinase. The GFP:Hip1r-CC+THATCH $_{(355-961)}$ fusion protein is found entirely in the membrane fraction after differential centrifugation (data not shown). Domain analysis of Hip1r, in addition to experiments with the endogenous protein, demonstrated that when Hip1r was not localized to the plasma membrane it was not phosphorylated, suggesting that Hip1r is phosphorylated at the plasma membrane. Moreover, expression of the only ENTH domain of epsin, which localizes exclusively to the plasma membrane, in epsin null cells allows for Hip1r phosphorylation and restores Hip1r to plasma membrane punctae (Chapter 2, this study; Brady and O'Halloran, unpublished). It is possible that phosphorylation of the protein could occur in the

cytoplasm prior to Hip1r association with the membrane. This potential scenario could be tested by analysis of samples obtained from subcellular fractionation experiments.

Identification of the unidentified kinase is important for further analysis of the phosphorylation of the protein and its role in Hip1r function. In the current study, I have shown that in addition to epsin, the central coiled region of the Hip1r protein, specifically the first 261 amino acids of the coiled-coil domain, is required for phosphorylation. It is conceivable that the entire coiled-coil region is necessary for appropriate protein folding and subsequent phosphorylation. However, analysis of the primary structure of the Hip1r protein reveals numerous phosphorylation sites. In particular, I have identified a single potential Casein Kinase II site conserved between the yeast, mammalian and *Dictyostelium* Hip1r orthologs. This site is located in the region of the coiled domain which was deleted in the FLAG: Hip1r-1/3CC+THATCH $_{(616\text{-}961)}$ fusion protein. To determine if these particular amino acids are the phosphorylated residues, the conserved sequence could be mutated by site-directed mutagenesis and the mutated protein could be introduced into *Dictyostelium* cells as a FLAG-tagged fusion protein. Examination of lysates from expressing cells by SDS-PAGE and immunoblotting with anti-FLAG antibodies would clarify whether these specific amino acids have a role in Hip1r phosphorylation. Interestingly, Casein Kinase II is an essential protein in *Dictyostelium* as deletion of the gene is lethal (Kikkawa et al., 1992). The kinase is expressed during both growing and developmental phases of the amoeba life cycle, making it an appealing candidate for the unidentified kinase acting upon Hip1r.

4.1.3 Epsin Acts Upstream of Hip1r

The dependence upon epsin for Hip1r localization and phosphorylation suggests epsin acts upstream of Hip1r. This hypothesis is strengthened by the observation that loss of Hip1r does not affect epsin localization to plasma membrane punctae and does not appear to influence epsin function in *Dictyostelium*. If this were true, I would expect the absence of both Hip1r and epsin to result in phenotypes similar to single Hip1r null cells as opposed to having a synergistic effect. In order to test this hypothesis, I generated a double mutant that harbored deletions in both Hip1r and epsin genes. I found that the resulting phenotypes of the Hip1r/epsin double mutant were indistinguishable from phenotypes described for single Hip1r or epsin null mutants. For example, the Hip1r/epsin null mutant did not display any gross abnormalities in clathrin or F-actin organization. In developing cells, the Hip1r/epsin double mutant formed fruiting bodies comparable to that of wild type but produced spores with abnormal morphology and reduced viability, similar to those of single Hip1r mutant spores. Taken together, these results reinforce the idea that epsin acts upstream of Hip1r in *Dictyostelium*.

The generation of a Hip1r/epsin double mutant provides another important tool for analysis of clathrin associated proteins in *Dictyostelium*. The Hip1r/epsin double mutant cell line could be used to examine the localization and function of additional accessory proteins for further characterization of factors thought to interact with both Hip1r and epsin during growth and development.

4.2 HIP1R IS REQUIRED FOR THE PRODUCTION OF VIABLE SPORES IN *DICTYOSTELIUM*

I have shown that Hip1r is required for the wild type morphology and viability of spores in *Dictyostelium* cells. Wild type spores are elliptical in shape and the spore coats consists of three layers--an outer protein layer, an middle layer composed of organized cellulose and an inner layer adjacent to the plasma membrane. Transmission electron microscopy images revealed the middle layer of the Hip1r null spores lacked organized cellulose. While Hip1r null cells were able to secrete necessary spore coat proteins and synthesize cellulose, the organization of cellulose within the spore coat was defective and the spores were unable to withstand adverse conditions. I propose that the inability to organize the cellulose leads to the abnormal morphology and reduced viability of the Hip1r null spores. A very recent publication characterizes a spore coat assembly pathway involving the formation of a molecular complex necessary for cellulose organization (Metcalf et al., 2007). If Hip1r participates in this particular pathway, the disorganization of the cellulose in the mutant spores may be a result of the absence of Hip1r. Collaboration with research groups that specializes in *Dictyostelium* developmental processes would enable a more detailed examination of the defects in the Hip1r mutant spore coat and enhance our understanding of Hip1r contribution to spore shape and viability. For example, does Hip1r have a role in the secretion of the recently-described molecular complex that is necessary for cellulose organization? Does Hip1r influence the formation of the molecular complex? Is the cellulose synthase enzyme properly positioned at the prespore membrane in Hip1r null cells?

Epsin null spores also exhibit the round spore morphology although the phenotype is less severe (Brady and O'Halloran, unpublished). Furthermore, the Hip1r/epsin double mutant cell line produces spores of similar shape and exhibit a viability rate comparable to that of single Hip1r null spores. I postulate that it is the mislocalization of Hip1r in epsin null cells that leads to the aberrant spore morphology observed in cells lacking epsin expression. If so, this would predict that the epsin spore coat would also have similar defects in cellulose organization. To test this possibility, the ultra-fine structure of the epsin null spore coat should be examined for similar defects in cellulose organization.

4.3 PARTICIPATION OF HIP1R AND EPSIN IN CLATHRIN INDEPENDENT EVENTS

In this study I have described the interaction between *Dictyostelium* Hip1r and another clathrin adaptor, epsin. Furthermore, I present evidence supporting a newly identified role for Hip1r orthologs, and for that matter, clathrin adaptors, during developmental processes. Characterization of Hip1r orthologs in other systems has focused on the role of the protein during clathrin-mediated events (Yang et al., 1999; Engqvist-Goldstein et al., 1999, 2000, 2004; Carreno et al., 2004). However, the potential involvement of Sla2/Hip1 family members in clathrin-independent pathways has yet to be addressed. While the evidence presented in the current study does not eliminate the contribution of Hip1r to clathrin function, the possible involvement of Hip1r and epsin in clathrin-independent processes cannot be ruled out. Recent work with mammalian epsin has demonstrated that the protein participates in clathrin-independent

endocytosis at the plasma membrane (Chen and De Camilli, 2005; Sigismund et al., 2005). Conceivably, Hip1r and epsin could function in clathrin-independent events in both growing and developing *Dictyostelium* cells. In growing cells, the lack of widespread colocalization between clathrin and Hip1r but extensive colocalization between Hip1r and epsin may be indicative of divergent pathways. In developing cells, particularly during spore formation, Hip1r and epsin may have evolved specialized functions that do not require an association with clathrin structures. While clathrin has been shown to be necessary for fruiting body formation, it does not appear to have a direct role in spore coat formation as determined by analysis of prespore vesicles (Niswonger and O'Halloran, 1997; Srinivasan et al., 1999). In contrast, both Hip1r and epsin are necessary for proper spore morphology and viability (This study; Brady and O'Halloran, unpublished). It is attractive to speculate that Hip1r and epsin may participate in regulatory endocytic and fusion events at the plasma membrane of prespore cells that function independent of clathrin. For example, Hip1r and epsin could have a role in positioning of the cellulose synthase enzyme in the plasma membrane during spore coat formation. Similarly, Hip1r and epsin may be part of the secretory machinery required for vesicle fusion with the plasma membrane during development and spore coat synthesis. These vesicles could contain spore coat proteins and/or factors necessary for the organization of cellulose and/or protein complexes within the spore coat. On the other hand, Hip1r and epsin could form part of an as-yet-unknown endocytic complex that functions to relay signals from neighboring cells to coordinate sporulation and coat formation. Moreover, the two proteins could share a dual function during the amoeba life cycle. During growth, Hip1r and epsin may participate in clathrin-dependent

processes. However, at the onset of the developmental program, the two proteins could function independent of clathrin during sporulation to produce robust spores with elliptical morphology. Regardless of whether the proteins function with or without clathrin during amoeba growth and development, the relationship between Hip1r and epsin described in this study warrants further investigation in *Dictyostelium* as well as other organisms.

4.4 THE ROLE OF HIP1R IN *DICTYOSTELIUM* CLATHRIN-MEDIATED ENDOCYTOSIS

Members of the Sla2/Hip1 family of proteins have been demonstrated to act during clathrin-dependent events in other systems. While the data presented in this study may not directly link *Dictyostelium* Hip1r to clathrin, indirect evidence supports its role as a clathrin adaptor. First, endogenous Hip1r co-localizes with epsin at punctae on the plasma membrane. In other systems, epsin is a known clathrin adaptor and in *Dictyostelium*, the epsin ortholog extensively colocalizes with clathrin (Wendland et al., 2002; Brady and O'Halloran, unpublished). Moreover, preliminary triple staining experiments by Rebecca Brady, another graduate student in the O'Halloran lab, show colocalization of all three proteins--epsin, clathrin and Hip1r--at the plasma membrane of cells (Brady and O'Halloran, unpublished). Second, in the absence of clathrin, Hip1r localization is perturbed. Specifically, rather than localizing at the plasma membrane in the form of punctae, endogenous Hip1r exhibits a "polarized" localization on one side of cell cortex (Figure 4.1A-B).

Experiments with cytochalasin A have revealed that F-actin is required for the scission, but not the assembly, of clathrin structures at the plasma membrane of *Dictyostelium* cells (Heuser and O'Halloran, unpublished results). Yeast Sla2p and mammalian Hip1r have been shown to bind and colocalize with F-actin (Yang et al., 1999; Engqvist-Goldstein et al., 1999). Furthermore, both orthologs have been postulated to act as molecular links between the endocytic machinery and F-actin during vesicle scission (Kaksonen et al, 2003; Engqvist-Goldstein et al., 2004). Although *Dictyostelium* Hip1r contains an actin-binding domain, the endogenous protein does not appear to colocalize with F-actin (data not shown). However, results from non-ionic detergent extraction of lysates and high salt buffer suggest an association of the protein with cytoskeletal factors. In addition, depolymerization of F-actin drives the total pool of cellular Hip1r to the cell cortex, suggesting some type of interaction between the two proteins (Figure 4.1C-D). A more detailed analysis of Hip1r and its association with F-actin is necessary to determine the extent of the contribution each protein makes to clathrin-mediated endocytosis in *Dictyostelium*. For example, the THATCH domain of Hip1r could be expressed as a fusion protein and used *in vitro* to test for actin binding in F-actin pelleting assays. Also, the role of Hip1r during cellular processes involving dynamic cytoskeletal rearrangements, such as chemotaxis or phagocytosis, could be assessed.

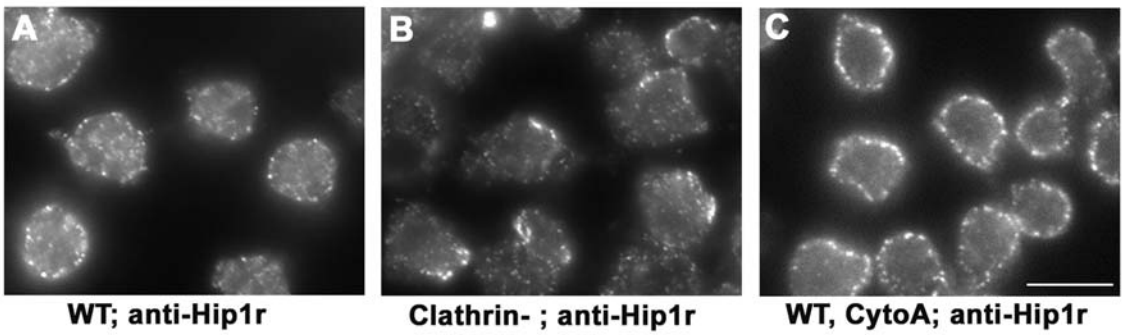

Figure 4.1 The localization of Hip1r requires clathrin and an organized cytoskeleton. (A-B) Wild type and clathrin null cells were fixed and stained with anti-Hip1r antibodies. In wild type cells (A, WT), Hip1r localized as membrane punctae around the periphery of the cell, with some cytoplasmic staining. In clathrin null cells (B, Clathrin-), Hip1r was not distributed in even punctae at the plasma membrane but appeared clustered on one side of the cell periphery. (C). Loss of functional F-actin restricts Hip1r punctae to the cellular cortex. Wild type cells were treated with cytochalasin A, an F-actin depolymerizing drug, then fixed and stained with anti-Hip1r antibodies. Endogenous Hip1r was localized almost exclusively to the cortex of the cell (C). Scale bar, 10 μM.

How does this study of *Dictyostelium* Hip1r expand the body of knowledge regarding clathrin-dependent processes and membrane trafficking events? The identification of a previously unidentified interaction between two clathrin adaptors, Hip1r and epsin, strengthens the idea that clathrin accessory proteins must not only work together with clathrin, but with each other to accomplish successful vesicle formation. Second, this study reinforces the concept that phosphorylation provides a means of regulating the localization and function of clathrin associated proteins. Lastly, this study demonstrates clathrin accessory proteins play an important role during multicellular development of organisms.

How does role of *Dictyostelium* Hip1r in cellular processes compare with other members of the Sla2/Hip1 family? Conceivably, more complex organisms may have evolved a more specialized role for the Hip1r orthologs and thus the function of the Hip1r protein in *Dictyostelium* may offer a glimpse into the evolutionary past eukaryotic membrane processes.

CHAPTER 5
EXPERIMENTAL PROCEDURES

5.1 Materials and Methods

5.1.1 Strain and Cell Culture

Dictyostelium discoideum wild type strains Ax2 and DH-1, Hip1r null and epsin null mutant strains were cultured in HL-5 nutrient media on Petri dishes at 20°C. Null cells were supplemented with 5ug/ml blasticidin. Strains expressing GFP constructs were also supplemented with 10ug/ml G418. Double mutant strains were grown in FM media (Formedium™; Norwich, England) or HL-5 and supplemented with 5ug/ml blasticidin.

5.1.2 Cloning of *hipA* and Polyclonal Antibody Generation

The amino acid sequence of mouse Hip1r and yeast Sla2p (accession numbers W82687 and Z22811, respectively) were used to search the *Dictyostelium* genome database (www.dictybase.org) using the BLAST search program (tBALSTn). Predicted protein sequences were analyzed using the DNAStar Megalign program (DNAStar, Inc. Madison, WI, USA).

Polyclonal antisera were raised against the approximately 20 kD carboxyl-terminal portion of Hip1r. This protein was expressed from plasmid pGEX2T-THATCH. The plasmid was constructed by amplifying this region from *Dictyostelium* cDNA via Polymerase chain reaction (PCR) using primers 5'-CCCGGGGGATGCTGCAAACTCATTATTGGCC-3' and 5'-

GAATTCTTGATTTTCGTCATATTGTTTCTTTC-3'. The product was cloned into the XmaI and EcoRI sites of the plasmid pGEX2T which situates the 3' end of the *hipA* gene downstream of the Glutathione-S-Transferase (GST) gene, thus generating an expression plasmid for a GST-C-terminal Hip1r fusion protein. pGEX2T-THATCH was transformed in *Escherichia coli* strain BL21 for large scale protein purification. Purification of the fusion protein was accomplished as described previously (Vithalani et al., 1998). The purified GST-Hip1r protein was used to generate polyclonal anti-Hip1r antibodies in rabbits (Cocalico Biologicals, Reamstown, PA, USA). For immunostaining experiments, the polyclonal antibodies were affinity-purified according to the manufacturer's directions using an affinity column of GST-C-terminal Hip1r fusion protein made with the GST Orientation kit (PIERCE, Rockford, IL, USA).

5.1.3 Immunofluorescence Microscopy

Growing cells were harvested, adjusted to $2x10^6$ cells/ml, plated on coverslips, and allowed to adhere at 20°C for 15 minutes. After a brief wash in PDF buffer (20 mM KCl, 11 mM K_2HP0_4, 13.2 mM KH_2PO_4, 1 mM $CaCl_2$ and 2.5 mM $MgSO_4$, pH 6.4) cells were fixed by submerging the coverslip in 2% formaldehyde in PDF buffer at room temperature for 15 minutes followed by permeabilization with 100% methanol at -20°C for 5 minutes. Cells were washed briefly in PDF buffer and processed for immunostaining.

For immunostaining, fixed cells on coverslips were blocked with 3% BSA in PBS solution for 15 minutes at 37°C and then incubated with affinity-purified anti-Hip1r antibodies [1μg/ml] or affinity-purified anti-clathrin antibodies [4 μg/ml] for 90 minutes

at 37°C. Coverslips were washed in PBS followed by incubation secondary antibody [30μg/ml], Texas Red-X conjugated Goat-anti-Rabbit IgG (Molecular Probes, Eugene, OR, USA) for 60 minutes at 37°C.

For F-actin staining, cells were fixed by submerging the coverslips in 2% formaldehyde in PDF buffer at room temperature for 15 minutes followed by permeabilization with 0.025% TX-100 in PDF buffer for 15 minutes. Cells were washed briefly in PDF buffer and processed for immunostaining with affinity-purified anti-Hip1r antibodies as described. Cellular F-actin was stained by incubating the fixed cells with Texas-Red conjugated phalloidin (Molecular Probes, Eugene, OR, USA) at a concentration suggested by the manufacturer for 25 minutes at room temperature. After rinsing in PBS, coverslips were mounted with mounting media onto slides and allowed to dry.

Images were taken on an inverted NIKON microscope TE2000 (NIKON Instruments, Dallas, TX) with 100x 1.4NA PlanFluor objective and Quantix camera (Roper Scientific, AZ, USA) controlled by Metamorph software (Universal Image Corp., PA, USA) and processed with Adobe Photoshop software (Adobe, San Jose, CA, USA).

5.1.4 SDS-PAGE Analysis

For gel samples, lysates of growing cells were harvested at a concentration 1 x 10^6 cells/ml. After boiling with SDS-containing sample buffer, lysates were loaded onto 7.5% or 10% SDS-polyacrylamide gels, electrophoresed and transferred to nylon membranes. After blocking with 3% nonfat milk in Tris buffered saline (TBS), membranes were incubated with polyclonal antibodies [1:1000], washed with TBS and

then incubated with goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase [1:5000] (Southern Biotech, Birmingham, AL, USA). Membranes were developed using the Supersignal® West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Rockford, IL, USA).

5.1.5 Phosphatase Treatment, Differential Centrifugation and Triton-X-100 Fractionation

To assess protein phosphorylation, cells were collected and resuspended in 1x NEB 3 buffer (New England Biolabs, Beverly, MA, USA) containing protease inhibitors diluted to the manufacturer's instructions (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO, USA). To lyse, cells were pelleted at 1500 x *g* for 3 minutes at 4°C and resuspended at a concentration of 2 x 10^6 cells/ml in 1x NEB 3 buffer containing protease inhibitors plus 0.5% TX-100. The resuspension was divided into test and control samples. CIP, Calf Intestinal Alkaline Phosphatase, (New England Biolabs, Beverly, MA, USA) was added to both samples and Okadaic Acid [10 ng/ml] (Sigma-Aldrich, St. Louis, MO, USA) was added to control samples to inhibit CIP activity. Samples were incubated at 37°C for 25 minutes and analyzed by SDS-PAGE (7.5% polyacrylamide gel) and western blotting.

For differential centrifugation, approximately 8 x10^8 cells were collected, washed with Isolation Buffer [10 mM MES (pH 6.5), 50 mM KAc (pH 6.5), 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.02% NaN_3], and resuspended to 4 x10^7 cells/ml in Isolation buffer with protease inhibitors diluted to the manufacturer's instructions (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO, USA). Cells were lysed by passing cells through two pieces of Osmonics (GE Osmonics, Trevose, PA, USA) polycarbonate

membrane (pore size: 5 µm) in a Gelman Luer-Lock-style filter (Gelman Sciences, Ann Arbor, MI, USA). The cell lysates were centrifuged at 3000 x *g* for 10 min at 4°C. The resulting low speed supernatants were ultracentrifuged at 100 000 x *g* for 60 min at 4 °C to generate a high-speed supernatant and a high-speed membrane pellet. Resulting fractions were analyzed via SDS-PAGE and western blotting.

To assess TX-100 fractionation, cells were harvested by centrifugation at 3000 x *g* for 5 minutes at 4°C. Cells were resuspended in MES buffer (20 mM MES (pH 6.5), 2 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT) containing protease inhibitors diluted to the manufacturer's instructions (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St. Louis, MO, USA), washed with Isolation Buffer, and resuspended in MES buffer and protease inhibitors plus 0.5% TX-100 to lyse. Lysates were spun at 16,060 x *g* at 4°C for 30 seconds to separate the TX-100 soluble and insoluble fractions. After collecting the soluble fraction, the insoluble fraction was resuspended in MES buffer in the same volume. Fractions were analyzed by SDS-PAGE and western blotting.

5.1.6 Disruption of the *hipA* Gene by Homologous Recombination

PCR was used to amplify genomic sequences flanking and within the coding region of the *Dictyostelium hipA* gene. The 5' upstream region of the *hipA* gene was amplified using the primers 5'-GATGACAGAGTTTGAAGCAATTGTCC-3' and 5'-GCAGCTTGTTGTTGTTGTAATTGTAAATTTGG-3'. The 3' downstream region of the gene was amplified using the primers 5'-CGTGTCGAAAAGGGTAAAACAAGTGATGG-3' and 5'-CGGTTTAAAAAAGTTACCATCAAGGC-3'. Each PCR fragment was initially cloned

into the vector pCR2.1 (Invitrogen) and then subcloned into the plasmid pSP72-BSR which carries a blasticidin resistance gene (Wang et al., 2002). The resulting plasmid, pSP72BSR-HipKO, was linearized with SacI and XhoI and introduced by electroporation into Ax2 cells. For electroporation, 5 x10^6 cells in 100 µl of buffer H-50 (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4) was mixed with 5 µg of linearized DNA and electroporated using a Bio-Rad Gene Pulser (75 kv, 25 µF) (Bio-Rad, Hercules, CA, USA). Each transformation reaction was diluted into HL-5 with 5 µg/ml blasticidin and plated into six 96-well plates. Resulting clonal lines were selected and screened by western blotting with anti-Hip1r antibodies. Replacement of the *hipA* gene by integration of the blasticidin cassette was confirmed by PCR analysis.

5.1.7 Disruption of the *hipA* and *epnA* Genes by Homologous Recombination

PCR was used to amplify genomic sequences flanking and within the coding region of the *Dictyostelium hipA* gene. The 5' upstream region of the *hipA* gene was amplified using the primers 5'-CTCGAGTGCTGAAATTTTTACATCCAACC-3' and 5'-AAGCTTGAAGGGTGGGTGGGTTTACG-3'. The 3' downstream region of the gene was amplified using the primers 5'-GAGCTCGCCAAATCCGTATCCAATGC-3' and 5'-GAATTCCCATTTACTTTCCGGAGAAATCTCG-3'. Each PCR fragment was initially cloned into the vector pCR2.1 (Invitrogen) and then subcloned into the plasmid pSP72-pyr which carries a pyrimidine biosynthetic gene (Wang et al, 2002). The pSP72-pyr plasmid is derived from pSP72-BSR and the pRH130 vector. The resulting plasmid, pSP72-pyr-HipKO, was linearized with XhoI and EcoRI and introduced by electroporation into DH1 cells. For electroporation, 5 x10^6 cells in 100 µl of buffer H-50

(20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4) was mixed with 5 µg of linearized DNA and electroporated using a Bio-Rad Gene Pulser (75 kv, 25 µF) (Bio-Rad, Hercules, CA, USA). Each transformation reaction was diluted into FM Media (Formedium™: Norwich, England) with and plated into six 96-well plates. Resulting clonal lines were selected and screened by western blotting with anti-Hip1r antibodies.

PCR was used to amplify genomic sequences flanking and within the coding region of the *Dictyostelium epnA* gene. The 5' upstream region of the *epnA* gene was amplified using the primers 5’ TTAAAAAAGGTAAAGATGCAGTATTG 3’ and 5’ TTGGAAATTTGGTGTTGCTGGTG 3’. The 3' downstream region of the gene was amplified using the primers 5’ AATCAAAGTGGTGCGAATAGAAATAC 3’and 5’ AATGATGATAGTAAAACTGATGGTAGAAG 3’. Each PCR fragment was initially cloned into the vector pCR2.1 (Invitrogen) and then subcloned into the plasmid pSP72-BSR which carries a blasticidin resistance gene (Wang et al., 2002). The resulting plasmid, pSP72-BSR-EpsinKO, was linearized with HindIII and EcoRI and introduced by electroporation into the Ax2 wild type strain or Hip1r null cells derived from the DHI wild type strain. For electroporation, 5×10^6 cells in 100 µl of buffer H-50 (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4) was mixed with 5 µg of linearized DNA and electroporated using a Bio-Rad Gene Pulser (75 kv, 25 µF) (Bio-Rad, Hercules, CA, USA). Each transformation reaction was diluted into HL-5 media with 5 µg/ml blasticidin or FM Media with 5 µg/ml blasticidin and plated into six 96-well plates. Resulting clonal lines were selected and screened by western blotting with anti-epsin or anti-Hip1r antibodies.

5.1.8 Generation of Green Fluorescent Protein (GFP) Constructs

The NH_2-terminal portion (amino acids 1-354) of Hip1r was amplified via PCR from *Dictyostelium* cDNA using the primers 5'-AAGCTTGGATGACAGAGTTTGAAGCAATTGTCCATAAAG-3' and 5'-GGTACCCGTTTGACGAATGAATACAGG-3'. The PCR product was initially cloned into pCR2.1 (Invitrogen). The plasmid was digested with HindIII and KpnI to release the Hip1r coding region and the fragment was cloned into the HindIII sites of pTxGFP (Levi et al., 2000) to generate an NH2-terminal Hip1r GFP fusion protein GFP:Hip1r-$ANTH_{(1-354)}$. The NH_2-terminus plus the central portion of the protein (amino acids 1-753) was amplified via PCR using the primers 5'-AAGCTTGGATGACAGAGTTTGAAGCAATTGTCCATAAAG-3' and 5'-GGTACCTCTCTTTGATTTTGCGGCCAATAATG-3' with *Dictyostelium* cDNA template. The PCR product was cloned into the pCR2.1 plasmid (Invitrogen) and then subcloned into the pTxGFP vector via the HindIII and KpnI restriction sites to generate a COOH-terminal deletion Hip1r protein fused to GFP (GFP:Hip1r-$ANTH+CC_{(1-753)}$). The central coiled region plus COOH-terminal THATCH domain of the Hip1r protein (amino acids 355-961) was cloned into pTxGFP after PCR amplification of *Dictyostelium* cDNA using the primers 5'-CTCGAGGGCAACAACAACAACTTTTACAACAACAACAGC`-3' and 5'-TCTAGATTGATTTTCGTCATATTGTTTCTTTC-3'. The PCR product was initially cloned into the pCR2.1 vector (Invitrogen) and digested with XhoI and XbaI to release the fragment and place into the same sites of the pTxGFP vector thus generating an NH_2-terminal deletion mutant (GFP: Hip1r-$CC+THATCH_{(355-961)}$).

To generate a full length epsin and GFP chimera, *Dictyostelium* cDNA was amplified via PCR with the 5'-TGGAGACTATGATTAAAAGTTATATTAAAAAAGGTAAAGATGCAGTATTGAA TACACCAGAAATTGAAAGAAAGGTTAG 3' and 5'-GCAGATCCCATGCTATTAGTATTTCTATTCGC 3'. The coding region of epsin subcloned into pCR2.1 (Invitrogen) and subsequently ligated into the pTxGFP vector using the KpnI and EcoRV restriction sites. To generate the epsin ENTH domain fused to GFP, the DNA encoding amino acids 1-333 was amplified via PCR using the primers 5' TGGAGACTATGATTAAAAGTTATATTAAAAAAGGTAAAGATGCAGTATTGAA TACACCAGAAATTGAAAGAAAGGTTAG 3' and 5' GGTCGACTTCTTCCGCCAG 3' and pCR2.1 containing full length epsin as a template. The epsin (1-333) PCR product was subcloned into the pCR2.1 vector. pCR2.1-Epsin (1-333) was then cut with EcoRI, blunted, and cloned into the EcoRV site pTxGFP, making pTX- pCR2.1-Epsin(1-333):GFP.

5.1.9 Generation of FLAG-tagged Constructs

The central coiled region plus THATCH domain of the Hip1r protein (amino acids 754-961) was cloned into pTxFLAG (kind gift from Tom Egelhoff) after PCR amplification of *Dictyostelium* cDNA using the primers 5'-CTCGAGGGCAACAACAACAACTTTTACAACAACAACAGC-3' and 5'-TCTAGATTGATTTTCGTCATATTGTTTCTTTC-3'. The PCR product was initially cloned into the pCR2.1 vector (Invitrogen) and digested with XhoI and XbaI to release the fragment and place into the same sites of the pTxFLAG vector thus generating an

NH_2-terminal deletion mutant (FLAG: Hip1r-CC+THATCH$_{(355-961)}$). The COOH-terminal portion of the central Hip1r coiled domain plus the THATCH domain (amino acids 616-961) was cloned into pTxFLAG after PCR amplification of *Dictyostelium* cDNA using the primers 5'-CTCGAGGGATGGATATTTTCTCTGCGGTCAATACAG-3' and 5'- TCTAGATTGATTTTCGTCATATTGTTTCTTTC-3'. The PCR product was initially cloned into the PCR2.1 vector and digested with XhoI and XbaI to release fragment and place into the same sites of the pTxFLAG vector thus generating the construct FLAG:Hip1r-1/3CC+THATCH$_{(616-961)}$.

5.1.10 Plasma Membrane Staining of Live *Dictyostelium* Cells

Live Ax2, Hip1r null and epsin null cells expressing the GFP: Hip1r-CC+THATCH$_{(355-961)}$ construct were aliquoted into 2-well microscopy chambers (Nalge-Nunc, International; Rochester, NY) and allowed to attach for 15 minutes. Media was aspirated and replaced with PDF buffer. Cells were incubated at 22°C for approximately 2 hours. To view colocalization of the GFP: Hip1r-CC+THATCH$_{(355-961)}$ chimera and the plasma membrane, the steryl dye, FM4-64 (Invitrogen, Molecular Probes) was diluted in low fluorescence media to a concentration of [1 μg/mL] and added to the cell chambers. Images of live cells were captured on an inverted NIKON microscope TE2000 (NIKON Instruments, Dallas, TX) with 100x 1.4NA PlanFluor objective and Quantix camera (Roper Scientific, AZ, USA) controlled by Metamorph software (Universal Image Corp., PA, USA) and processed with Adobe Photoshop software (Adobe, San Jose, CA, USA).

5.1.11 F-Actin Depolymerization of *Dictyostelium* Cells

Wild type Ax2 cells were harvested and adjusted to $2x10^6$ cells/ml, plated on coverslips, and allowed to adhere at 20°C for 15 minutes. Cells were incubated with Cytochalasin A (Calbiochem; La Jolla, CA USA), diluted to [10 μM/mL] in PDF buffer and incubated at 22°C for 45 minutes to allow for actin depolymerization. After a brief wash in PDF buffer, cells were fixed by submerging the coverslip in 2% formaldehyde in PDF buffer at room temperature for 15 minutes followed by permeabilization with 100% methanol at -20°C for 5 minutes. Cells were washed briefly in PDF buffer and processed for immunostaining with affinity-purified anti-Hip1r antibodies as described above.

Images were taken on an inverted NIKON microscope TE2000 (NIKON Instruments, Dallas, TX) with 100x 1.4NA PlanFluor objective and Quantix camera (Roper Scientific, AZ, USA) controlled by Metamorph software (Universal Image Corp., PA, USA) and processed with Adobe Photoshop Software (Adobe, San Jose, CA, USA).

5.1.12 Measurement of Pinocytosis and Secretion

Fluid phase endocytosis was measured by uptake of FITC-dextran [2 mg/ml], as described by Ruscetti et al. (Ruscetti, et al., 1994). Log-phase cells were harvested and 1-2 x 10^6 cells were grown overnight in suspension cultures until the cells reached a titer of 3-4 x 10^6 cells/ml. For pinocytosis assays, FITC-dextran was added to a final concentration of [2 mg/ml] and incubated at 20°C. For secretion studies, cells were incubated with FITC-dextran for 2 hours at 20°C before taking samples from the cultures. Fluorescence of internalized FITC-dextran was quantified using a VersaFluor

Fluorometer (Bio-Rad, Hercules, CA, USA) equipped with the filter set for FITC, a 485-485 nm excitation filter and a 515-525 nm emission filter.

5.1.13 Development of Fruiting Bodies

For development of fruiting bodies, cells were harvested and washed twice with starvation buffer (20 mM MES, 0.2 mM CaCl2, 2 mM MgSO4) and plated onto either non- nutrient agar plates (1% Noble agar; Difco Laboratories, Inc.) at 20°C or seeded on SM-5 plates containing a lawn of bacteria (*Escherichia coli* B/R) and grown at 20°C. Fruiting bodies were harvested and images were taken with either a Zeiss microscope Semi SR with 1.2x or 2.0x objectives controlled by NIH image software or with an inverted NIKON microscope TE2000 (NIKON Instruments, Dallas, TX) with 100x 1.4NA PlanFluor objective and Quantix camera (Roper Scientific, AZ, USA) controlled by Metamorph software (Universal Image Corp., PA, USA). Images were adjusted using Adobe Photoshop software.

5.1.14 Spore Viability Assays

The viability of wild type, Hip1r null spores, epsin null spores and Hip1r/epsin mutant spores were assayed by harvesting approximately 2 x 10^8 cells and plating onto non-nutrient agar plates. Cells were grown at 20°C for 4 days to allow development into fruiting bodies. Spores were harvested by inverting plates and tapping gently to collect spores on the lid of the petri dish. The collected spores were suspended into spore buffer (40 mM KH_2PO_4, 20 mM KCl, 2.5 mM $MgCL_2$), washed twice by centrifugation at 12,500 x *g* for 2 minutes at room temperature and counted with a hemocytometer. Spores

were either treated with heated to 45°C for 20 minutes or incubated with 0.5% or 0.1% NP40 detergent (Sigma-Aldrich, St. Louis, MO, USA) in spore buffer for 5 minutes or incubated with spore buffer alone for 5 minutes. Spores were washed and plated in triplicate onto SM-5 agar plates containing a lawn of bacteria (*Escherichia coli* B/R) and grown for 14 days at 20°C. Viability of spores was assessed by counting the number of clear plaques formed on the bacterial lawns.

5.1.15 Spore Fixation and Staining

Approximately 2 x 10^8 growing wild type, Hip1r null cells, epsin null cells and Hip1r/epsin mutant cells were harvested, washed and plated on non-nutrient agar plates. Cells were grown at 20°C for 4 days to allow for spore formation. Spores were collected by inverting plates and tapping gently, resuspended into and then washed twice with spore buffer and placed on coverslips treated with poly-L-lysine. Excess buffer was removed and the adhered spores were air dried for 15 minutes.

The presence of cellulose in harvested spores was detected by staining with 1 μg/ml Calcofluor (Sigma-Aldrich, St. Louis, MO, USA).

For immunostaining experiments, spores were fixed by the addition of 2% paraformaldehyde in PDF buffer to the coverslips with harvested spores. Coverslips were incubated for 15 minutes at room temperature. Spores were washed briefly in PDF buffer and blocked with 3% BSA in PBS solution for 15 minutes 37°C. To detect SP70, 100μl of a [1:200] solution of anti-SP70 antibodies (gift of Dr. Richard Gomer) were incubated with fixed spores for 90 minutes at 37°C. Spores were washed in PBS

followed by incubation with 60 μl of a [30μg/ml] secondary antibody, Texas Red-X conjugated Goat-anti-Rabbit IgG (Molecular Probes, Eugene, OR, USA) for 60 minutes at 37°C. Spores were washed in PBS and mounted onto clean slides with mounting media and allowed to dry.

Images were taken on an inverted NIKON microscope TE2000 (NIKON Instruments, Dallas, TX) with a 100x 1.4NA PlanFluor objective and a Quantix camera (Roper Scientific, AZ, USA) controlled by Metamorph software (Universal Image Corp., PA, USA) and processed with Adobe Photoshop software.

5.1.16 Electron Microscopy

To examine spores under electron microscopy, ~2 x 10^8 wild type and Hip1r null spores were harvested, washed and resuspended in 2% gluteraldehyde (EM grade) in spore buffer and incubated for 1 hour at 20°C. After this fixation, the spores were washed in spore buffer and examined either by Scanning Electron Microscopy (SEM) or by Transmission Electron Microscopy (TEM).

SEM spore samples were dehydrated through a graded ethanol series to 100% ethanol, critical point dried in a Tousimis Samdri-790, and coated with a gold-palladium alloy. Specimens were visualized with a Philips 515 scanning electron microscope and images were processed with Adobe Photoshop software.

TEM spore samples were embedded in 1% agarose, post fixed in 2% osmium tetroxide for 2 hours and dehydrated through a graded ethanol series to 100% ethanol. The ethanol was replaced with epoxy resin and samples were baked. Spore samples were sectioned at less than 100 nm thickness on a RMC MT6000-XL ultramicrotome, stained

with uranyl acetate and lead citrate, and viewed on a Philips EM208 TEM. Images were processed with Adobe Photoshop software.

5.2 Plasmids and Cell Lines

Table 5.1 Plasmids and Cell Lines used in this Study

Plasmids	Description
pGEX2T-THATCH	Hip1r THATCH domain cloned in bacterial expression vector for protein purification and polyclonal antibody production
pSP72BSR-HipKO	1.1 kb 5' fragment of 5 UTR and coding region of *hipA* and 1kb 3' fragment of 3' UTR of *hipA* and coding region flanking the blasticidin resistance gene cassette to replace the *hipA* gene
pSP72-pyr-HipKO	1 kb fragment of 5' UTR of *hipA* gene and 1kb fragment of 3' UTR of *hipA* gene flanking the pyrimidine biosynthetic gene to replace the *hipA* gene

pSP72-BSR-EpsinKO (by Rebecca Brady)	Fragment of 5' UTR of *epnA* gene and (by fragment of 3'UTR of *epnA* gene flanking the blasticidin resistance gene cassette to replace the *epnA* gene
pTxGFP:Hip1r-ANTH$_{(1-354)}$	The first 354 amino acids of Hip1r, including the ANTH domain. C-terminal GFP tag. G418 resistance.
pTxGFP:Hip1r-ANTH+CC$_{(1-753)}$	The first 753 amino acids of Hip1r, including the ANTH plus central coiled-coil domains. C-terminal GFP tag. G418 resistance.
pTxGFP: Hip1r-CC+THATCH$_{(355-961)}$	Last 606 amino acids of Hip1r, containing the central coiled-coil plus THATCH domains. N-terminal GFP tag. G418 resistance.
pTxGFP:Epsin (by Rebecca Brady)	Full length epsin. N-terminal GFP tag. G418 resistance.
pTX- pCR2.1-Epsin $_{(1-333)}$:GFP. Rebecca Brady)	The ENTH domain of epsin. N-terminal (by GFP tag. G418 resistance.
pTxFLAG: Hip1r-CC+THATCH$_{(355-961)}$	Last 606 amino acids of Hip1r, containing the central coiled-coil plus THATCH domains. N-terminal FLAG tag. G418 resistance.

pTxFLAG:Hip1r-1/3CC+THATCH$_{(616-961)}$	Last 345 amino acids of Hip1r, including the last 261 amino acids of the central coiled-coil plus THATCH domains. N-terminal FLAG tag. G418 resistance.
pTxCLC:GFP	*Dictyostelium* clathrin light chain, NH2-terminal GFP tag; G418 resistance. (Wang et al., 2003)

Cell Lines	Description
Ax2	Wild type axenic strain; grows in HL-5 media supplemented with 0.6% penicillin/streptomycin (P/S)
DH1	Derived from Ax3 wild type axenic strain, with the pyrimidine biosythetic gene deleted. Uracil oxotroph; growth in HL-5 with 0.6% P/S and FM minimal media.
Hip1r null (4F6)	Derived from Ax2 parent strain, *hipA* gene disrupted by blasticidin cassette; growth in HL-5 supplemented with 5μg/mL blasticidin.
Hip1r null (3D4)	Derived from DH1 parent strain, *hipA* gene disrupted by pyrimidine biosynthetic gene of pSP72-pyr-HipKO. Growth in FM media.

Epsin null (5B4) (by Rebecca Brady)	Derived from Ax2 parent strain, *epnA* gene disrupted by blasticidin cassette; Grows in HL-5 supplemented with 5μg/mL blasticidin.
Epsin null (E4B1) (by Rebecca Brady)	Derived from DH1 parent strain, *epnA* gene disrupted by blasticidin cassette; growth in HL-5 supplemented with 5μg/mL blasticidin.
Hip1r/epsin null (3H11)	Derived from DH1 parent strain. *hipA* and *epnA* genes deleted using the pSP72-pyr-HipKO and pSP72-BSR-EpsinKO plasmids. Growth in FM media supplemented with 5 μg/mL blasticidin.
Clathrin HC null (5E2) (by Maria Niswonger)	Derived from Ax2 parent strain. *chcA* gene is deleted; growth in HL-5 supplemented with 5 μg/mL blasticidin. (Niswonger and O'Halloran, 1997).

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Vita

Shannon Lea Repass, the eldest daughter of Marsha Ross and the late Randy Ross and eldest granddaughter of the late Pauline and Sam Ross was born in Tyler, Texas on July 17, 1970. After attending Robert E. Lee High School in Tyler, Texas, she entered the University of Texas at Austin in Austin, Texas where she received the degree of Bachelor of Science in Microbiology in 1994. After completing her undergraduate degree, she was employed as a research technician at The University of Texas at Austin and at the Texas Department of Health. In 1998, she was admitted to the Graduate School at Southern Methodist University in Dallas, Texas and earned a Master of Science degree in Biology in 2000 under the supervision of Dr. Jack Kennell. In August of 2000, she entered the Graduate School at The University of Texas at Austin and joined the research group of Dr. Terry O'Halloran in the section of Molecular, Cell and Developmental Biology in 2001. Her work focused on the characterization of proteins involved in membrane trafficking events in the amoeba, *Dictyostelium discoideum*.

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